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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US92/09433 <b>(22) International Filing Date:</b> 4 November 1992 (04.11.92)  <b>(30) Priority data:</b> 787,826 5 November 1991 (05.11.91) US  <b>(71) Applicant:</b> STATE UNIVERSITY OF NEW JERSEY - RUTGERS [US/US]; 377 Hoes Lane, Piscataway, NJ 08855 (US).  <b>(72) Inventors:</b> MESSING, Joachim ; 17 Neuville Drive, Somerset, NJ 08873 (US). FISHER, Hans ; 216 North 3rd Avenue, Highland Park, NJ 08904 (US) UEDA, Takashi ; 166 Capricorn Drive #13, Somerville, NJ 08876 (US).		<b>(74) Agent:</b> WHITE, John, P.; Cooper & Dunham, 30 Rockefeller Plaza, New York, NY 10112 (US).  <b>(81) Designated States:</b> JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> A METHOD OF OBTAINING HIGH METHIONINE-CONTAINING CORN SEEDS, AND USES THEREOF  <b>(57) Abstract</b>  <p>This invention provides methods of obtaining corn seeds having a methionine content of greater than 1.39 percent by weight of total amino acid composition, and which can provide greater than 36 percent of the methionine nutritional requirements of poultry. The high-methionine corn is obtained as the progeny of a parental corn line selected to produce corn containing high levels of methionine-rich 10 kDa zein. Alternatively, the high-methionine corn seed is produced by a corn plant that has been genetically transformed with an expression construct which comprises a seed-functional promoter operably linked to gene encoding a methionine-rich zein protein, an operably linked DNA sequence capable of enhancing the functioning of the promoter, an operably linked DNA sequence capable of stabilizing the zein gene mRNA, and an operably linked DNA sequence capable of enhancing the translation of the zein gene mRNA. The invention further provides a method of determining whether an introduced gene is expressed in maize endosperm tissue, and it also provides a purified antibody specific for a maize high-methionine protein.</p>		

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**A METHOD OF OBTAINING HIGH METHIONINE-CONTAINING CORN SEEDS, AND USES THEREOF**

This invention was made with support under Grant No. 85ER13367 from the U.S. Department of Energy. Accordingly, the U.S. government has certain rights in  
5 the invention.

**Background of the Invention**

Throughout this application various publications are  
10 referenced by Arabic numerals. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosure of these publications is hereby incorporated by reference into  
15 this application to describe more fully the art to which this invention pertains.

Maize (Zea mays) is a nutritious food substance commonly used for feeding livestock. However, it is deficient with respect to certain nutrients, one of which is the  
20 amino acid methionine (1, 2). Additional methionine is usually introduced into the livestock diet through dietary supplements, but this procedure is costly and hence economically undesirable. Improving the  
25 nutritional balance of maize is thus an important commercial objective.

**1. Amino acid storage in corn**

Mature maize seeds contain very low levels of free amino acids and, therefore, most of their amino acids are  
30 derived from seeds as hydrolysates, either by germination to feed the growing seedling or by digestion with respect to human and livestock consumption (3). In this respect, proteins serve as a storage form of amino acids, and the

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genes encoding these proteins exercise control over the total balance of amino acids by their primary structure and by the level of their accumulation during seed development. One also can think about storage proteins as a sink for assimilated nitrogen produced during the photosynthetic period of the life cycle of the plant. The different locations of synthesis and storage require an interesting signalling pathway and transport system between leaves, where photosynthesis occurs, and flowers, where storage proteins are synthesized after fertilization is successful. Furthermore, the differential expression of storage proteins amplifies any imbalances of amino acid accumulation (for review see (4)). An interesting example of such quantitative variability is the expression of the high-methionine storage protein gene of maize.

The majority of storage proteins in maize are extracted with ethanol and, when their amino acid composition is determined, one can readily recognize that certain essential amino acids are underrepresented (1, 2). Tryptophan, lysine, and methionine are low, while leucine is very high. The amino acid proline is also very abundant and, besides glutamine, contributes to the general name prolamine, a group also referred to as zeins in maize.

Regulatory loci that affect the balance of amino acids in the maize kernel have been identified. The loci operate by affecting the accumulation of a subset of storage proteins. The best known regulatory locus is probably opaque-2 which affects lysine content (1). Although it was first thought that this regulation was accomplished by increasing the accumulation of lysine-rich proteins,

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it turned out that the primary effect was the failure to express lysine-poor proteins. Recently, the opaque-2 gene has been cloned and its product, belonging to the leucine zipper family of transcription factors, has been shown to control transcription of a certain subset of zein genes by binding to the promoter region (5). The failure of expressing a subset of these genes reduces the amount of proteins poor in lysine, thereby proportionally enhancing the level of those that are higher in lysine. However, to overcome the reduced protein levels in opaque-2 kernels, breeders have introduced other regulatory loci, also called modifiers, to restore protein levels by deregulating other storage protein genes (6).

A regulatory locus that acts on the amino acid balance without noticeable reduction of storage protein synthesis is Zpr10/(22) (7). This locus causes the increase of the level of a minor zein protein that is rich in methionine residues. The methionine accumulates mainly into two zein gene products--one of 15kDa and one of 10kDa relative molecular weight. Both proteins are encoded by single genes and are present in all standard inbreds looked at so far. The 15kDa zein gene contains about 12% methionine codons, while the 10kDa zein gene contains about 23% methionine codons. The regulatory gene Zpr10/(22), however, selectively causes the increased accumulation of the 10kDa zein protein during endosperm development.

## 2. Zein proteins and genes

Zeins are the alcohol-soluble fraction of storage proteins in maize (Zea mays). They constitute more than 50% of the total endosperm proteins at seed maturity.

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Zeins consist of a group of heterologous hydrophobic proteins, which are classified according to their molecular weight, as determined on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) into subclasses with Mr of 27, 22, 19, 16, 15, and 10kDa (8, 9). Based on structural similarities, they are also  
5 classified into  $\alpha$ -(22 and 19 kDa),  $\beta$ -(15kDa),  $\gamma$ -(16 and 27kDa), and  $\delta$ -(10kDa) zeins (10). Zeins are encoded by a complex multigene family of over 100 gene members (11-13) and regulated in a tissue- and developmental stage-specific manner. Their expression is confined to  
10 triploid (3n) endosperm tissue and starts at a specific stage [around 12 days after pollination (DAP)] during endosperm development (14). Furthermore, the onset of elevated zein gene expression coincides with the genome amplification process starting at this particular stage  
15 in endosperm development (15).

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Summary of the Invention

This invention provides a method of obtaining corn seeds or kernels having a methionine content of greater than 1.39 percent by weight of the total amino acid composition of the corn seeds or kernels. This invention also provides a method for providing greater than 36 percent of the methionine nutritional requirements of poultry. This invention further provides a method of improving the growth performance of poultry. This invention further provides a corn plant resulting from a genetic cross comprising high zein protein-containing seeds. This invention further provides a recombinant nucleic acid molecule consisting essentially of (1) a sequence encoding a zein protein, (2) a sequence which when present in the molecule is capable of functioning as a promoter of transcription of the sequence encoding the zein protein, and (3) an exogenous sequence capable of (a) enhancing the functioning of the promoter sequence, (b) stabilizing the transcription product of the zein protein-encoding sequence, or (c) enhancing the translation of the transcription product of the zein protein-encoding sequence, the promoter sequence and the exogenous sequence being so positioned with respect to the sequence encoding the zein protein that the zein protein is expressed when the recombinant nucleic acid molecule is introduced into a suitable host cell. This invention still further provides a method of determining whether an exogenous nucleic acid molecule will be expressed in maize endosperm tissue. Finally, this invention provides a purified antibody specific for a maize high-methionine protein.

**Brief Description of the Figures****Figure 1**

**Northern blot analysis of RNA isolated from maize plant tissues and tissue cultures.** Total RNA was isolated from endosperm (16 DAP), embryo (16 DAP), root and leaf tissues of an A636 maize plant, as well as from A636 endosperm tissue culture and BMS leaf tissue culture. RNA samples were fractionated in a formaldehyde-agarose gel, transferred onto a filter, and hybridized to the zein-specific probes as indicated: 5  $\mu$ g of total RNA from each maize tissue was analyzed (left); 20  $\mu$ g of total RNA from maize tissue cultures was analyzed (right).

**Figure 2**

**Quantitative comparison of zein message levels in endosperm culture.** Of total RNA isolated from the maize tissues indicated, 2  $\mu$ g was blotted on a filter in a slot-blot apparatus. The filter was hybridized to probes specific for the 10-, 15-, and 27-kDa zein genes and for 17S rDNA. Hybridization intensity on the autoradiogram was quantitated by densitometry.

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**Figure 3A**

**S1 nuclease mapping of the 10- and 27-kDa zein genes.** Diagrammatic representation of the 5' coding regions of the 10- and 27-kDa zein genes. The coding sequences of the two zein genes are indicated by solid boxes. The probes used for the 10- and 27-kDa zein genes are the 188-bp *Ava*II-*Ban*I fragment and the 343-bp *Rsa*I-*Hpa*II fragment, respectively. The 5' ends of the noncoding strands of these fragments were radiolabeled with  $\gamma$ -<sup>32</sup>P-ATP. The sizes of the protected bands are also shown. The trinucleotide ATC found at the transcription initiation site for the two zein genes is indicated together with TATAA sequences and the ATG initiation codon.

**Figure 3B**

**S1 nuclease mapping of the 10- and 27-kDa zein genes.** S1 nuclease reaction products were fractionated on 6% polyacrylamide gels containing 8 M urea. Maxam and Gilbert (16) sequencing reactions of the probes were run along with the S1 nuclease reaction products (not shown). Higher counts of the S1 nuclease reaction products for the RNA samples from endosperm culture were loaded in the gels for the comparison. The nucleotide sequences of the coding strands in the region of the protected bands and the location of the bands (arrows) are shown at the left margin of each figure.

**Figure 4A**

**Transient expression of chimeric genes in endosperm protoplasts.** Diagrammatic representation of the chimeric constructs used in the electroporation experiments. pFFCAT contains the CAT gene coding sequence fused to the CaMV35S promoter with a duplicated enhancer and the

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CaMV35S terminator in pFF19 (17). The pZ10(-1076/+42)CAT and pZ27(-1042/+61) contain 1.1-kb 5' flanking sequences of the 10- and 27-kDa zein genes, respectively. End points with respect to the cap sites are designated in parentheses. Promoter-less CAT construct  $\phi$ -CAT was used as a negative control. pFFGUS (17), containing the GUS coding sequences fused to the CaMV35S gene promoter and terminator, was cotransfected with each CAT construct to serve as an internal standard for electroporation.

10 Figure 4B

Transient expression of chimeric genes in endosperm protoplasts. CAT enzyme assay of transfected endosperm protoplasts. Lane 1, no plasmid DNA added; lane 2, 150  $\mu$ g of promoter-less  $\phi$ -CAT added; lane 3, 150  $\mu$ g of pZ10(-1076/+42)CAT added; lane 4, 150  $\mu$ g of pZ27(-1042/+61)CAT added; lane 5, 25  $\mu$ g pFFCAT added. For each assay, 100  $\mu$ g of protein extract was used.

Figure 5

20 Northern blot analysis of RNA isolated from maize plant tissue and cultured cells. Total RNA was isolated from endosperm (16 DAP), root and leaf tissue of an A636 maize plant, as well as from A636 endosperm and leaf tissue-derived BMS cultures. Five micrograms of RNA sample from each tissue were fractionated in a formaldehyde-agarose gel, transferred onto a filter, and hybridized to a  $^{32}$ P-labelled O2 cDNA probe. It is demonstrated here that the endosperm-specific expression of the O2 gene is maintained in the A636 endosperm culture.

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Figure 6

A. A diagrammatic representation of chimeric constructs used to test the effect of the O2 overexpression on the

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two zein promoters;  $\phi$ -GUS, the promoter-less GUS reporter equipped with the CaMV35S terminator sequence (35ST); pZ-4GUS, the GUS-35ST placed under the regulation of the 0.9-kb 22-kDa Z-4 zein promoter; pZ27GUS, the GUS-35ST sequence placed under the regulation of the 1.1-kb 27-kDa zein promoter.

5     B. A diagrammatic representation of the O2 overexpression constructs which are cotransfected with the zein promoter-GUS constructs shown in panel A; pFF02+, a full length O2 cDNA placed under the regulation of the CaMV35S promoter with duplicated enhancer elements (pCaMV35S+) and its terminator (35ST); pFF02m, an internal deletion clone of the O2 cDNA (lacking the sequence encoding a  
10     part of the O2 protein including the bZip domain) placed under the regulation of the pCaMV35S+ and the 35ST.

15     C. Relative GUS activities derived from the transiently transformed maize endosperm protoplasts which have been cotransfected with the zein promoter-GUS and the O2 overexpression constructs. The non-specific GUS activity derived from the  $\phi$ -GUS construct was subtracted from those derived from zein promoters for each  
20     cotransfection.

#### Figure 7

25     A. A diagrammatic representation of chimeric constructs used to test the effect of O2 overexpression on the O2-binding cis sequence from the Z-4 promoter;  $\phi$ -CAT, the promoter-less CAT reporter equipped with the CaMV35S terminator sequence (35ST); pCRCAT, a truncated (-90) CaMV35S promoter (pCR) fused to the CAT-35ST construct; pCR50+CAT, a chimeric promoter consisting of the pCR and a 5-mer of a 33-bp upstream sequence from the 22-kDa zein Z-4 promoter (containing the O2 binding sequence) fused to the CAT-35ST construct; pCR50mCAT, a chimeric promoter  
30

consisting of the PCR and a 5-mer of a 33-bp upstream sequence from the 22-kDa zein pML-1 promoter fused to the CAT-35ST construct. These constructs were cotransfected into maize endosperm protoplasts together with the O2 overexpression cassettes shown in Figure 6, panel B.

5 B. Relative CAT activities derived from the transiently transformed maize endosperm protoplasts. The non-specific CAT activity derived from the  $\phi$ -CAT construct was subtracted from those derived from zein promoters for each cotransfection. The CAT activities derived from chimeric CAT constructs were standardized to that from  
10 pCRCAT cotransfected without plasmid DNA.

### Figure 8

Construction of pUM5010. The cloning steps involved in the construction of pUM5010 are outlined. Plasmid  
15 descriptions: pZ4, a 1.1-kb 5' flanking sequence of the 22-kDa Z-4 zein gene cloned into pUC119 vector; pZ40, a 33-bp upstream sequence of the Z-4 promoter containing the O2 binding sequence cloned into pBS (KS) vector; pZ450, the 33-bp sequence polymerized five times in pBS  
20 (KS) vector; pCRZ450, the 5-mer of the 33-bp sequence fused to a truncated (-90) CaMV35S promoter in pBS (KS) vector; pZ10H, a 3.9-kb genomic subclone of the 10-kDa zein gene in pUC119 vector; pZ10HB, creation of a BamHI site at the 5' end of the coding sequence by site-  
25 directed mutagenesis; pUM5010, the 1.2-kb BamHI-DraI fragment from the pZ10HB (containing the entire coding and the 3' flanking sequences of the 10-kDa zein gene) fused to the chimeric promoter in pCRZ450. Abbreviations in the 10-kDa zein coding sequence: ATG, initiation  
30 codon; TAG, stop codon; AATAAA, polyadenylation signal.

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Figure 9

Response of zein promoters to an exogenously added phytohormone, abscisic acid (ABA). Other than the trans-regulation by Opaque-2 proteins, it is also found that ABA can differentially regulate promoter function of zein genes. As seen in this figure, exogenously added ABA (50 $\mu$ M) enhances the promoter activity of the 27-kDa zein gene while it suppresses that of the 10-kDa zein gene. In addition, the promoter activity of the CaMV35S promoter is not affected by ABA. It is speculated here that different classes of endogenous zein genes may respond differentially to the endogenous ABA level in the kernel which is known to increase during seed maturation. Constructs used in the figures; lane 1, CAT enzyme purified from *E. coli*; lanes 2 and 3, no plasmid controls; lanes 4 and 5, promoter-less  $\phi$ -CAT; lanes 6 and 7, pZ27(2.0)CAT consisting of a CAT reporter placed under the regulation of the 2.0-kb 27-kDa zein promoter and CaMV35S terminator (35ST); pZ10(1.1)CAT consisting of the chimeric CAT reporter gene placed under the regulation of the 1.1-kb 10-kDa zein promoter; lanes 10 and 11, pFFCAT consisting of the chimeric CAT reporter gene placed under the regulation of the CaMV35S promoter with duplicated enhancer. Endosperm protoplasts transfected with each construct were cultured for two days in the absence (-) or presence (+) of 50  $\mu$ M ABA before enzymatic assays were performed.

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**Detailed Description of the Invention**

5 The plasmid pUM5010 was deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC Accession No. 68644.

10 This invention provides a method of obtaining corn seeds or kernels having a methionine content of greater than 1.39 percent by weight of the total amino acid composition of the corn seeds or kernels, which comprises crossing a paternal inbred corn line containing the  
15 Zpr10/(22) locus with a maternal inbred corn line lacking the Zpr10(22) locus and selecting for F1 hybrid seeds containing methionine at greater than 1.39 percent by weight of the total amino acid composition of the F1 hybrid seeds or kernels. The paternal inbred corn line  
20 may be BSSS-53. The maternal inbred corn line may be M017, W23 or W22.

25 This invention also provides a method for providing greater than 36 percent of the methionine nutritional requirements of poultry which comprises feeding the poultry corn having a methionine content of at least 1.39 percent by weight of the total amino acid composition of the corn.

30 In this invention, the corn may have a methionine content of at least 3.8 percent. Also in this invention, the



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poultry may be chickens. The corn may be in the form of seeds or kernels, or in the form of cornmeal.

5 This invention also provides a method of improving the growth performance of poultry which comprises feeding the poultry corn having a methionine content of at least 3.8 percent by weight of the total amino acid composition of the corn.

10 This invention further provides a corn plant resulting from a genetic cross comprising high zein protein-containing seeds having a methionine content of at least 1.39 percent by weight of the total amino acid composition of the seeds. The seeds may have a methionine content of at least 3.8 percent. Also, the genetic cross  
15 may be a reciprocal cross between a Zpr10/(22)-containing maize inbred and a normal female maize inbred lacking Zpr10/(22). The Zpr10/(22)-containing inbred may be BSSS-53. The normal female maize inbred may be M017, W23 or W22.

20 This invention further provides a recombinant nucleic acid molecule consisting essentially of (1) a sequence encoding a zein protein, (2) a sequence which when present in the molecule is capable of functioning as a  
25 promoter of transcription of the sequence encoding the zein protein, and (3) an exogenous sequence capable of (a) enhancing the functioning of the promoter sequence, (b) stabilizing the transcription product of the zein protein-encoding sequence, or (c) enhancing the  
30 translation of the transcription product of the zein protein-encoding sequence, the promoter sequence and the exogenous sequence being so positioned with respect to the sequence encoding the zein protein that the zein

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protein is expressed when the recombinant nucleic acid molecule is introduced into a suitable host cell.

As used in the subject invention, an "exogenous sequence" means a nucleic acid sequence, from maize or any other source, which would not naturally be situated next to the zein protein-encoding sequence of the subject invention as it is so situated in the recombinant nucleic acid molecule of the subject invention.

One example of the recombinant nucleic acid molecule of the subject invention is a molecule having these three distinct, non-overlapping sequences: (1) a sequence encoding a zein protein; (2) a sequence which is capable of functioning as a promoter of transcription of the sequence encoding the zein protein, and (3) an exogenous sequence capable of (a) enhancing the functioning of the promoter sequence, (b) stabilizing the transcription product of the zein protein-encoding sequence, or (c) enhancing the translation of the transcription product of the zein protein-encoding sequence. Another example of the recombinant nucleic acid molecule of the subject invention is a molecule having these two distinct, non-overlapping sequences: (1) a sequence encoding a zein protein; and (2) a sequence which is capable of functioning as a promoter of transcription of the sequence encoding the zein protein, and which contains an exogenous sequence capable of (a) enhancing the functioning of the promoter sequence, (b) stabilizing the transcription product of the zein protein-encoding sequence, or (c) enhancing the translation of the transcription product of the zein protein-encoding sequence. In the latter example, the exogenous sequence may contain a portion of, or all of, the sequence capable

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of functioning as a promoter. These examples are by no means intended to limit the scope of the subject invention.

5 In the recombinant nucleic acid molecule of the subject invention, the sequence encoding the zein protein may be a naturally occurring zein gene. The naturally occurring zein gene may be a maize zein gene, and this maize zein gene may be a high-methionine maize zein gene. The high-methionine maize zein gene may be a 10kDa high-methionine maize zein gene.

10

The sequence encoding the 10-kDa high methionine maize zein gene may be obtained by cleaving it from the plasmid pUM5010 using a suitable restriction enzyme.

15 In the recombinant nucleic acid molecule of the subject invention, the exogenous sequence may comprise an O<sub>2</sub>-binding region of a maize 22kDa promoter. The exogenous sequence may be a multimer of O<sub>2</sub>-binding regions of maize 22kDa promoters. Such multimers show an enhancing effect superior to that of monomers. The multimer of O<sub>2</sub>-binding regions of maize 22kD promoters may comprise 5 copies of the O<sub>2</sub>-binding region. The recombinant molecule of the subject invention may be the molecule designated pUM5010.

20  
25 The O<sub>2</sub>-binding region may be obtained by cleaving it from pUM5010 using a suitable restriction enzyme.

30 In the recombinant nucleic acid molecule of the subject invention, the exogenous sequence may comprise an ABA regulatory element. The exogenous sequence may be a multimer of ABA regulatory elements. Such multimers show an enhancing effect superior to that of monomers. The

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multimer of ABA regulatory elements may comprise 5 copies of the ABA regulatory element.

5 In the recombinant nucleic acid molecule of the subject invention, the exogenous sequence may comprise the 3' region of the B gene of the 27kDa maize gene. The 3' region of the B gene of the 27kDa maize gene may be fused to the 3' end of the sequence encoding the zein protein.

10 This invention further provides a method of increasing the concentration of zein protein in a plant, which comprises treating the plant so as to incorporate the nucleic acid molecule of the subject invention into the genome of the plant.

15 In the method of the subject invention, the plant may be maize, rice, soybean, alfalfa, barley or wheat.

20 As used in the subject invention, the incorporation of the nucleic acid molecule may comprise microinjecting the molecule into cells of the plant. Alternatively, the incorporation of the nucleic acid molecule may comprise firing the molecule with a particle gun into cells of the plant. Alternatively, the incorporation of the nucleic acid molecule may comprise contacting embryonic culture  
25 cells of the plant with the molecule.

30 This invention further provides a genetically engineered corn plant having high zein protein-containing seeds and a methionine content of at least 1.39 percent by weight of the total amino acids present in the seeds comprising the recombinant nucleic acid of the subject invention. The methionine content of the high zein protein-

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containing seeds may be at least 3.8 percent by weight of the total amino acids present in the seeds.

5 This invention further provides a method of determining whether an exogenous nucleic acid molecule will be expressed in maize endosperm tissue which comprises introducing the exogenous nucleic acid molecule into cultured maize endosperm protoplasts suspended in a suitable buffer; culturing the resulting maize endosperm protoplasts containing the exogenous nucleic acid molecule; and detecting expression of the nucleic acid molecule by the maize endosperm protoplasts so as to thereby determine whether the nucleic acid molecule will be expressed in maize endosperm tissue. The exogenous nucleic acid molecule may be the recombinant nucleic acid molecule of the subject invention.

15 In the method of the subject invention, the introduction of the exogenous nucleic acid molecule may comprise performing electroporation on the protoplasts in the presence of the exogenous nucleic acid molecule.

20 In the method of the subject invention, the detection of expression may comprise isolating RNA from the cultured protoplasts containing the nucleic acid molecule; and determining the presence of RNA transcribed from the nucleic acid molecule in the RNA so isolated. Alternatively, the detection of expression may comprise performing an enzyme assay on cultured protoplasts containing the nucleic acid molecule, wherein the nucleic acid molecule encodes an enzyme having detectable activity, and detecting this activity of the enzyme. The enzyme may be chloramphenicol acetyltransferase (CAT) or  $\beta$ -glucuronidase (GUS).

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5 This invention further provides a purified antibody specific for a maize high-methionine protein. The antibody of the subject invention may be a monoclonal antibody, a murine antibody or a rabbit antibody. In the antibody of the subject invention, the maize high-methionine protein may be the maize 10kDa high-methionine protein.

10 Finally, this invention provides a method of determining the level of high-methionine protein produced by a maize strain which comprises preparing a protein-containing sample from the maize strain; contacting the sample with the antibody of the subject invention under conditions such that the antibody complexes with any high-methionine protein present in the sample for which the antibody is specific; and determining the amount of high-methionine protein present in any resulting complex.

20 This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

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## Experimental Details

### I. Maternal Effects on High Methionine Levels in Hybrid Corn

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#### 1. Summary

The inbred BSSS-53 (Maize Stock Center, University of Illinois, Urbana) containing the Zpr10/(22) factor on chromosome 4 that influences the levels of the high methionine 10kDa zein protein in maize encoded by the Zps10/(22) locus on chromosome 9 was crossed reciprocally with a line that lacks the Zpr10/(22) product. The amino acid composition of the prolamine fraction from progeny seeds of both reciprocal crosses and self-fed parents were determined. The level of protein-bound methionine in the two hybrids shows a maternal influence rather than a simple dosage effect as one would expect for a nuclear endosperm gene. The large margin of difference in protein-bound methionine of the two reciprocal hybrids provided an opportunity to test its nutritional significance by a feeding trial of one day old male Peterson's Arbor Acre chicks. Although the efficiency of weight gain per food consumed is the same in both experimental groups, the high methionine corn meal improved growth performance between 35% and 54%.

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#### 2. Introduction

Mature seeds contain very low levels of free amino acids and, therefore, most of their amino acids are derived from seeds as hydrolysates either by germination to feed the growing seedling or by digestion with respect to human and livestock consumption (3). In this respect, proteins serve as a storage form of amino acids, and the

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genes encoding these proteins exercise control over the total balance of amino acids by their primary structure and by the level of their accumulation during seed development. One also can think about storage proteins as a sink for assimilated nitrogen produced during the photosynthetic period of the life cycle of the plant. The different locations of synthesis and storage require an interesting signalling pathway and transport system between leaves where photosynthesis occurs and flowers where storage proteins are synthesized after fertilization is successful. Furthermore, the differential expression of storage proteins amplifies any imbalances in amino acid accumulation (for review see (4)). An interesting example of such a quantitative variability is the high-methionine storage protein gene of maize.

The majority of storage proteins in maize are extracted with ethanol and, when their amino acid composition is determined, one can readily recognize that certain essential amino acids are underrepresented (1, 2). Tryptophan, lysine, and methionine are low, while leucine is very high. The amino acid proline is also very abundant and, besides glutamine, contributes to the general name prolamine, a group also referred to as zeins in maize.

The abundant amino acids which are hydrophobic also determine the solubility of the majority of storage proteins in maize endosperm. When these proteins are then characterized either by their mobility or isoelectric focusing properties, it becomes apparent that they are represented by a multigene family (18). Therefore, the amino acid sequence of a single member has



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to be determined by cDNA cloning and sequencing (19). DNA cloning has also revealed that the size of the storage protein gene family is in the order of about one hundred genes. It has also revealed that the amino acid composition not only varies, but also allows one to divide the storage proteins into two types: those that  
5 are multicopy and those that are single copy (20).

Regulatory loci that affect the balance of amino acids in the maize kernel have been identified. The loci operate by affecting the accumulation of a subset of storage  
10 proteins. The best known regulatory locus is probably opaque-2 which affects lysine content (1). Although it was first thought that this regulation was accomplished by increasing the accumulation of lysine-rich proteins, it turned out that the primary effect was the failure to  
15 express lysine-poor proteins. Recently, the opaque-2 gene has been cloned and its product, belonging to the leucine zipper family of transcription factors, has been shown to control transcription of a certain subset of zein genes by binding to the promoter region (5). The  
20 failure of expressing a subset of these genes reduces the amount of proteins poor in lysine, thereby proportionally enhancing the level of those that are higher in lysine. However, to overcome the reduced protein levels in opaque-2 kernels, breeders have introduced other  
25 regulatory loci, also called modifiers, to restore protein levels by deregulating other storage protein genes (6).

A regulatory locus that acts on the amino acid balance without noticeable reduction of storage protein synthesis  
30 is Zpr10/(22) (7). This locus causes the increase of the level of a minor zein protein that is rich in methionine

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residues. The methionine accumulates mainly into two zein gene products, one of 15kDa and one of 10kDa relative molecular weight. Both proteins are encoded by single genes and are present in all standard inbreds looked at so far. The 15 kDa zein gene contains about 12% methionine codons, while the 10kDa zein gene contains about 23% methionine codons. The regulatory gene Zpr10/(22), however, selectively causes the increased accumulation of the 10kDa zein protein during endosperm development.

### 10 3. Materials and Methods

#### a. Inbreds.

BSSS-53 was used as a donor for Zpr10/(22) which has been shown to be a single Mendelian trait located on the short arm of chromosome 4 (7) and which is absent in inbred lines like M017 and W23 (Maize Stock Center, University of Illinois, Urbana) which were used as recipients. Therefore, these recipients were treated as zpr10/(22). All inbreds used contain the locus expressing the 10kDa zein gene. Since there may be other 10kDa zein genes that are expressed at various levels, the high-methionine gene was cloned (21) and mapped on the long arm of chromosome 9 close to the centromere and was named Zps10/(22) because it was able to respond to the regulatory locus Zpr10/(22) (7). To ensure reproducibility of the difference in methionine levels, ears were collected from about 500 plants for each genotype. All plants were hand-pollinated and grown in the greenhouse during the winter of 1988/1989 and in the field during the summer of 1989.

#### 30 b. Protein extraction.

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The prolamine fraction was extracted under reducing conditions to include both zein 1 and the zein 2 components as described previously (22).

c. Amino acid composition analysis.

5 The prolamine fraction was hydrolysed in liquid phase as described by Metzler et al. (23). Samples were compared to the appropriate internal standards using an Applied Biosystem Amino Acid Analyzer.

10 d. Feeding trial.

The same samples that were subjected to the amino acid composition analysis were used as a source for the corn meal in a fourteen day long feeding trial carried out in our animal facilities with one-day old male Peterson's Arbor Acre chicks.

15 The chicks were fed a highly methionine-deficient diet that derived its protein principally from isolated soy protein. This protein and the composition of the remainder of the diet have been previously shown to be  
20 deficient only in methionine (24-26). The National Research Council stated that the methionine requirement for chicks of the type used is 0.50% of the diet (27), and the control diet with zpr10/(22)/Zpr10/(22) corn  
25 provided only 0.18%. The high-methionine corn diet provided double that amount, or 0.38%.

4. Results

30 a. Amino acid composition of zeins with respect to the dosage of Zpr10/(22).

When Zpr10/(22) was crossed into different inbreds, it was not only found that all alleles of the Zps10/(22)

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locus encoding the structural gene for the 10kDa zein high methionine protein were responsive to Zpr10/(22), but also that the trans-acting mechanism occurred mainly at the translational or post translation level (28). Furthermore, the study also showed that at least one other regulatory gene can segregate and influence the accumulation of 10kDa zein mRNA levels independently of Zpr10/(22). Therefore, the accumulation of methionine in maize seeds appears to be a) variable among inbred lines, b) dependent on genetic factors that segregate as Mendelian factors, and c) unusually high in the inbred BSSS-53 because of the presence of at least two genetic factors in the same inbred.

All flowering plants reproduce by double fertilization, wherein one sperm fertilizes the egg and the other fertilizes the polar nuclei. In most cases, a second nucleus is fused in the second fertilization event leading to a triploid pre-endosperm nucleus with two maternal contributions and one paternal one. Therefore, different dosages of Zpr10/(22) can be found in the F1 depending on the parent. Mapping of Zpr10/(22) was accomplished by using high 10kDa zein protein levels as a phenotype (7). This analysis also showed a dosage dependence in the F1 hybrids with inbreds that are zpr10/(22). Such a dosage is typical for semidominant factors in endosperm.

Therefore, in the F1 of a hybrid between a plant carrying Zpr10/(22) and one carrying zpr10/(22), one would predict a 2:1 dose for the two reciprocal crosses, while one parent carries three doses and the other none. If BSSS-53 is used as a donor for Zpr10/(22), then any other trans-acting factor in BSSS-53 that increases 10kDa zein

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gene expression would follow the same dosage distribution. However, when corn meal was produced from such an F1 population, and the amino acid composition of total protein was analyzed (Materials and Methods, Part I), a different result than the one predicted would have emerged. When Zpr10/(22) was passed through the pollen, methionine levels were nearly the same as those for homozygous zpr10/(22)--0.73% and 0.75%, as shown in Table I, respectively. In comparison to these, the Zpr10/(22) parent, BSSS-53, had a relative level of 1.39%. This increase by a factor of about two is consistent with earlier comparisons made between BSSS-53 and other inbred lines (29). However, when Zpr10/(22) is passed through the female plant, the relative methionine level increases to 3.84%, nearly three times as high as in its homozygous state and more than five times as high as in the reciprocal cross.

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**TABLE 1**

**Amino Acid Composition of the Prolamine Fraction<sup>a</sup> from Progeny of Reciprocal Crosses of Inbreds With and Without Zpr10/(22).**

		<u>Genotype</u>			
	<u>Amino Acid<sup>b</sup></u>	<u>Z<sup>c</sup>/Z/Z</u>	<u>Z/Z/+</u>	<u>+/+/Z</u>	<u>+/+/+</u>
5	<u>Ala</u>	27.47 <sup>d</sup>	24.61	28.66	29.81
	<u>Leu</u>	24.15	21.57	23.71	23.32
	<u>Glx</u>	14.20	16.58	15.08	15.70
	<u>Asx</u>	6.21	6.26	7.11	6.39
	<u>Phe</u>	5.09	5.08	5.45	5.73
10	<u>Gly</u>	5.00	5.77	4.80	4.23
	<u>Val</u>	4.41	4.05	4.53	2.92
	<u>Tyr</u>	3.33	3.71	2.94	3.06
	<u>Ile</u>	3.40	2.53	2.88	3.08
	<u>Arg</u>	1.69	1.79	1.40	1.53
15	<u>Ser</u>	1.17	1.39	1.32	1.32
	<u>Thr</u>	1.41	1.58	0.51	1.12
	<u>His</u>	0.93	1.04	0.85	0.99
	<u>Met</u>	1.39	3.84	0.75	0.73
	<u>Lys</u>	0.15	0.19	nd <sup>e</sup>	0.08
	<u>Trp</u>	nd	nd	nd	nd

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<sup>a</sup>The prolamine fraction of mature seeds was extracted with ethanol as described previously (22); <sup>b</sup>Amino acids are listed in the order of their abundance; only primary amines were determined which do not include proline; essential amino acids are underlined Z=Zpr10/(22); the methionine values are highlighted in bold. <sup>c</sup>Z=Zpr10/(22) the female parent is contributing two doses to the triploid endosperm. <sup>d</sup>The amino acid composition of the prolamine fraction was carried out by liquid phase hydrolysis as described by Metzler, et al. (23) and is expressed in percent of total amino acids. <sup>e</sup>Not detectable.

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b. Availability of protein-bound methionine from two reciprocal hybrids.

This unusually high level of methionine in the maternal hybrid creates a very large difference of methionine levels between the two reciprocal crosses, and this difference could be of practical significance. Although lysine and tryptophan are the first limiting amino acids in corn, corn-based commercial feeds derive much of their amino acids from soybean meal, the major protein constituent of such diets. Thus, if high-lysine corn (opaque-2) is used in such a diet instead of normal corn, only the addition of extra synthetic methionine was shown to improve growth performance of chickens, indicating that enough lysine is usually supplied by the soybean protein (30). Nelson et al. (31) observed earlier that in homozygous floury-2 plants, not only the lysine and tryptophan content is increased but also the methionine content was about 50-70% higher. Therefore, diets with a reduced soybean meal content, and using floury-2 corn, have been shown to promote faster growth and better feed conversion efficiency in chickens than have diets with normal corn (32). In these experiments, lysine appears to be the limiting factor and the difference in methionine seemed to be too small to make a significant difference. Sugary-1 endosperm also increases methionine content, but again only to the same degree (33). Furthermore, in both cases of floury-2 and sugary-1, these loci have pleiotropic effects and not the same specific trans-acting function as Zpr10/(22).

Therefore, corn meal was used from the two reciprocal crosses that show the five-fold difference in methionine levels to set up two different feed rations (Materials

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and Methods, Part I). As can be seen in Table II, a soybean/corn composition was chosen that usually is supplemented with free methionine. Since it has been shown previously that this diet is complete except for methionine (24-26), the corn meal from the two reciprocal crosses was the only variable for the relative amounts of methionine. Even if other amino acid levels in the two corn meals differed, their margin had already been adequately covered by the soybean protein. From the recommended requirements of the National Research Council it appears that this high methionine corn diet reached about 75% of the chickens' optimal requirements (Materials and Methods, Part I). The two different diets were used to feed Peterson's Arbor Acre male one-day old chicks in duplicate groups of 5 per treatment. Body weight and food consumed were determined after 14 days. Although food utilization by each group was very similar, about 0.7, the growth performance with the high methionine corn diet was significantly different as shown in Table III, and shows a margin of improvement from 35% to 54%. This experiment shows that although the differential level of methionine is not large in biochemical terms, it is large enough to make a substantial nutritional difference. More importantly, it also shows that the methionine that accumulates in zeins is available in the diet and can replace free methionine for optimal growth.



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**TABLE II**

**Diet Composition For Feeding Trial<sup>a</sup> With Corn Meal From  
The Two Reciprocal Crosses Between Zpr10/(22) And  
Zpr10/(22)**

5	<b>Ingredient</b>	<b>Amount<sup>b</sup></b>
	Isolated soy protein <sup>c</sup>	15.0
	Mineral mixture	5.3
10	Cellulose (Solka Floc)	3.0
	Corn oil	3.0
	Vitamin mixture	0.4
	Corn meal <sup>d</sup>	73.3
15	Total	100.0

<sup>a</sup>Peterson's Arbor Acre male chicks were used in a 14 day feeding trial starting with one-day-old animals in duplicate groups of five per treatment; <sup>b</sup>the composition is expressed in percentage of the total diet; <sup>c</sup>the soy protein mixture contains 1.0 methionine and 0.6 cystine per 16% nitrogen; <sup>d</sup>corn meal was derived either from the Zpr10/(22)/Zpr10/(22)/+ or +/+/Zpr10(22) hybrids as described in Table I.

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**TABLE III**

**Growth Performance of Chicks Fed With Corn Meal From  
Progeny of Reciprocal Crosses of Inbreds With and Without  
Zpr10(22)**

5	Genotype	Body Weight <sup>a</sup>		Food Consumed	
		Group I	Group II	Group I	Group II
	+/+/Z <sup>b</sup>	141(28) <sup>c</sup>	164(24)	152 <sup>d</sup>	202
10	Z/Z/+ <sup>b</sup>	217(38)	220(26)	249	245
		Efficiency			
		Group I	Group II		
15	+/+/Z <sup>b</sup>	0.69 <sup>e</sup>	0.62		
	Z/Z/+ <sup>b</sup>	0.72	0.73		

<sup>a</sup>Body weight was determined in g after 14 days of growth starting with one-day-old animals ranging in weight from 35-40 g; <sup>b</sup>Z=Zpr10/(22); the female parent is contributing two doses to the triploid endosperm; <sup>c</sup>average weight is given in g for each group of five chicks and the standard deviation for each average weight is given in parentheses; <sup>d</sup>average amount of food consumed is given in g for each group; <sup>e</sup>the efficiency factor is the proportion of g weight gained per g of food consumed.

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### 5. Discussion

It has been shown that reciprocal crosses between inbreds that differ in the regulatory locus Zpr10/(22) not only show an unusual effect on the methionine levels in their progeny endosperm, but also show an unexpected impact on a typical corn/soybean diet for poultry. The discrepancies between 10kDa protein levels and protein-bound methionine levels arising from these studies and previous observations (7) could be explained by the reduced accumulation of the Zps10/(22) product and the onset of expression of "filler proteins" (with similar properties but lower methionine content) that are otherwise suppressed. Therefore, it will be useful to use BSSS-53 as a source of 10kDa zein antigen and to prepare specific antibodies to the Zps10/(22) product because this inbred is likely to be devoid of 10kDa "filler proteins". If the synthesis of other zein proteins of 10kDa relative molecular weight with different methionine content can be derepressed in the appropriate genetic background, they could be further characterized by cDNA cloning. With respect to such differential expression of 10kDa zeins, it is interesting that earlier findings of Zps10/(22) mRNA levels also did not reflect a simple dosage pattern (28). Of course, these measurements reflect different stages of endosperm development which can differ among inbreds and hybrids in their developmental window, while the amino acid composition was prepared from mature seeds. Nevertheless, after taking many measurements, it is apparent that Zps10/(22) mRNA levels also show a maternal effect in crosses with BSSS-53.

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Although maternal effects are usually associated with cytoplasmic traits, the F2 and backcross data involving Zpr10/(22) published earlier clearly indicate segregation of Zpr10/(22) rather than dependence on the origin of the cytoplasm (7). Maternal effects on gene expression that depend on nuclear genes have been observed in many organisms and have been referred to as imprinting (for review see (34)). In maize, maternal effects on endosperm nuclear genes are complicated by the different contribution of the female and male gametes. Therefore, the R-mottling phenotype described by Emerson (35) that affects anthocyanin synthesis in the aleurone layer of the endosperm typical for Indian corn was initially very difficult to interpret. When R was passed through the pollen, kernels of a recipient became mottled, while in the reciprocal cross, kernels became solidly colored. One also could argue that a certain threshold is required for full color. However, Kermicle (36) was able to use a TB<sup>A</sup> translocation to increase the copy number of R from the sperm and show the independence of the phenotype from dosage. If the phenotype is not dependent on dosage but is dependent on passage of the gene through a particular parent, two steps are required during the development of the gametophyte. An imprinting step and a revision step have to take place to distinguish the gene coming from either parent. It is interesting that these reversible modifications of genes must be reproduced during mitotic division by mechanisms different from the standard base pairing (37).

Although an interesting correlation between DNA methylation and differential activation of gene expression has been observed (38), this might well be a simple default mechanism and the actual memory may

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consist of sequence-specific DNA binding proteins. When the DNA is replicated during mitosis, one strand becomes temporarily hemimethylated. This indicates that methylation of the new strand is probably occurring soon after replication. Since DNA binding proteins can protect the DNA from methylation (39), it is quite conceivable to think of DNA methylation as a footprint of coated DNA. Conversely, once methylation has occurred, the binding specificity of proteins to DNA may change in turn. If this is true, the sequence-specific DNA binding proteins have to be replenished after each mitosis and their binding races against the methylation process. Differential expression of these DNA binding proteins during gametogenesis could then explain the difference in imprinting, and erasure of imprinting is also just a default. In this respect, it is interesting that many transcription factors are found throughout development while others are produced only during gene expression. With many of these factors acting as hetero- and homodimers, one can easily envision a new dimension of combinatorial controls.

In this case, the high methionine phenotype is also an endosperm trait, and therefore subject to the same ambiguity between imprinting and the threshold potential of two copies introduced by the female gamete. In either case, however, the unusually high levels of methionine in the hybrid with the maternal contribution of Zpr10/(22) are reminiscent of the general effect on heterosis observed in hybrid corn.

## II. Use of High Methionine Zein Plants

The improvement of the nutritional balance of corn is an interesting commercial project because it could save large amounts of money in expensive feed supplements (4).

5 One of these supplements is methionine, an essential amino acid. These experiments involve the study of a maize inbred BSSS-53 that was screened from over 100 lines for high methionine on the seedling level (40). It became clear that levels of methionine in mature kernels

10 of different common inbred lines varied due to the differential expression of a single gene which is present in all of them (41). This single gene does not encode an enzyme in the methionine biosynthesis pathway, but rather a storage protein, also called the 10kDa zein, which

15 accumulates in endosperm of maize kernels during seed development. When the primary structure of the protein was revealed by sequencing the corresponding cDNA, it was found that 23% of its coding information is in AUG methionine codons (21). . Therefore, the expression of

20 this zein gene during seed development serves as a methionine sink for the plant. A feeding trial of chickens, using progeny from two reciprocal crosses that differ dramatically in amounts of the 10kDa protein, showed a dramatic impact on the performance of growth and

25 feather quality indicating that the 10kDa protein is used as a methionine source by the animals (42). The five-fold difference in methionine was tested in a standard corn/bean diet without supplemented methionine by feeding Peterson's Arbor Acre male one day-old chicks in

30 duplicate groups of 5 per treatment. Food consumed and body weight were determined after 14 days. Although the efficiency of weight gain per food consumed is the same in both experimental groups, the high methionine corn

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meal improved growth performance between 35% and 54%. From the recommended requirements of the National Research Council, it appears that this high methionine corn diet reached about 75% of the chickens' optimal requirements.

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# 1. Genetic Basis of Quantitative Variability

In the last years, the basis of the quantitative variability of 10kDa zein expression among different  
10 inbred lines has been investigated. It is interesting that BSSS-53, the high methionine inbred line, contains a factor that boosts the expression of the 10kDa zein gene thereby leading to the enhanced accumulation of high methionine protein in BSSS-53 kernels. This factor is  
15 called Zpr10(22) because it regulates the expression of the 10kDa zein locus which is also called Zps10(22). Since these two loci are on different chromosomes and segregate as single Mendelian factors, Zpr10(22) encodes a product that acts in trans (7). Furthermore, using a  
20 10kDa cDNA probe and the high 10kDa RNA levels in BSSS-53 as a phenotype, it also became clear in the segregation analysis of Zpr10/(22) and Zps10/(22) that the high RNA phenotype is independently controlled from the high protein phenotype (28). Therefore, we assume that in  
25 addition to Zpr10/(22), other trans-acting factors might control the overexpression of the 10kDa zein gene. Furthermore, this segregation analysis then indicates that the product of Zpr10/(22) most likely acts on the translational or post-translational level.  
30 Overexpression of the RNA on the other hand could be regulated either on the transcriptional or post-transcriptional level. However, using nuclear run-off transcription experiments we found that post-

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transcriptional regulation is required for the increased steady state 10kDa RNA levels (28).

## 2. Significance of Trans-acting Factors For Transgenic Plants

5        These different steps of regulation of gene expression  
can be dissected into potentially useful cis and trans-  
acting factors. Since it was shown 1) the 10kDa  
methionine protein can be overexpressed and 2) the  
overexpressed amount can substitute methionine  
10       supplements without penalty in growth performance, it  
becomes attractive to engineer the regulation of the  
10kDa zein gene. To assess the efficacy of hybrid  
promoter and hybrid messages of the 10kDa zein mRNA  
before the generation of transgenic maize plants under  
15       conditions that are as close as possible to the natural  
tissue, a homologous expression system for maize  
endosperm genes was developed. Since the relevant  
developmental stage has been arrested in tissue culture  
suspension cells and since these cells can be efficiently  
20       converted into protoplasts at any time, transient  
expression experiments can be carried out very  
economically and with all tissue-specific factors usually  
present during normal seed development (43). Based on  
experiments carried out in this system with different cis  
25       and trans-acting elements, from genetic data, and from  
other precedents, it is possible to propose a combination  
of new elements for a mosaic 10kDa zein gene that could  
be used for the construction of transgenic maize plants  
that could reach the level of 10kDa zein expression  
30       necessary to reach the 100% optimal diet requirements  
without adding any free methionine supplement. Although  
one could propose a very complex mosaic gene, four



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examples are described here to demonstrate the principle of the approach disclosed herein. In all these cases, pairs of the cis-acting sites of the mosaic gene are matched with the corresponding trans-acting factors that positively enhances 10kDa zein gene expression. As used herein, the term "pair" means a cis-acting element and its corresponding trans-acting factor. Except for one case, these relevant trans-acting factors are naturally produced during endosperm development of all common inbreds. In these cases only the cis acting sites have to be engineered.

The first pair is the opaque-2 gene product present in all elite lines and its cis-acting sites. Absence of the opaque-2 product, which is a leucine-zipper type of DNA binding protein, causes the elimination of the transcription of a specific subset of zein genes. Other zein genes that lack the "opaque-2 box" are not affected. Recently, it has been shown in a homologous expression system (43) that introduction of such an "opaque-2 box" into a gene, where it usually is absent, leads to a specific increase in gene expression when the opaque-2 gene is expressed on a second plasmid. Furthermore, this effect is more dramatic if tandem copies of the restriction fragment containing the "opaque-2 box" are placed into the promoter region. If the restriction fragment in the tandem repeat is replaced by the equivalent one from the 22kDa zein promoter pML1, which differs in two positions from the canonical opaque-2 binding site, the trans-acting opaque-2 fails to show increased expression in our homologous expression system. Similarly to the pML1 gene, the 10kDa zein gene has at the same distance from the transcriptional start site a sequence that differs from the canonical opaque-2 binding

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site. Therefore, the 10kDa promoter also does not respond to the trans-acting opaque protein in the homologous expression system, which is in agreement with the observation that opaque-2 variants are not affected in their 10kDa zein levels. However, introduction of  
5 e.g. a fivemer of the "opaque-2 box" into the right position of the promoter of the 10kDa zein gene should render the 10kDa promoter sensitive to opaque-2 regulation. This can be tested in the homologous expression system and should lead to enhanced  
10 transcription of 10kDa zein message in transgenic plants.

The second pair is ABA (abscisic acid) and an ABA regulatory element absent in the 10kDa, but present in the 27kDa zein gene. The ABA regulatory element can be  
15 obtained by 1) isolating a genomic subclone containing the 27kDa zein gene containing the ABA regulatory element according to the method of Das and Messing (44), and 2) isolating from the subclone a 1,103-bp PvuI fragment containing the ABA regulatory element. Using the  
20 homologous expression system (43), preliminary data were obtained showing differences in the response of the 10kDa and 27kDa zein promoters to increased ABA levels. Again, the absence of such a responsive element in the 10kDa zein promoter, allows us to introduce such an element  
25 into the corresponding position of the 10kDa zein promoter and should similar like the opaque-2 responsive element lead to increased 10kDa zein transcription.

The third pair is related to post-transcriptional regulation. As shown recently, Zpr10/(22) shows the  
30 importance of post-transcriptional regulation (7, 42). However, it has two problems. First, it is absent in

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standard inbreds. Second, it is regulated by imprinting.

In contrast to the Zpr10/(22) responsive element in the 10kDa zein gene, the 27 kDa zein gene possesses a responsive element that seems not to be subject to segregating trans-acting factors. This became clear from dosage experiments described previously (44). The 27kDa zein gene is present in a tandem duplication in many inbred lines. This organization is also called the S allele, because homologous recombination products were found and isolated having the deletion of the duplication that differs in one retaining the first (A gene) or the other retaining the second (B gene) copy of the 27kDa zein gene (45, 46). It appears that the second gene copy is expressed at a higher level due to the accumulation of 2.5-fold more message. The 3' untranslated regions of the 10kDa zein gene could be exchanged with the one from the 27kDa B gene, making the 10kDa message relatively more stable due to a trans-acting factor that does not segregate.

The fourth pair is the yeast GAL cis-acting element and the trans-acting factor GCN4, a non-conventional involving a heterologous expression system. Similarly to the opaque-2 and "opaque-2 box" pair, for example, the yeast GAL cis- and trans-acting regulatory system can be introduced by cloning the cis-acting element into the 10kDa promoter region and the expression the trans-acting factor GCN4 under the 10kDa zein promoter from a different chromosomal location. In the latter case, the cis and trans-acting regulatory pair can actually be introduced into different transgenic plants and the overexpression tested in the hybrid.

### III. A Homologous Expression System For Cloned Zein Genes

#### 1. Summary

Expression of the genes encoding the 10-, 15-, and 27-kDa zeins is maintained in suspension cultures derived from developing endosperm tissue of maize (Zea mays L.). Although expression of these genes is reduced in endosperm cultures as compared with that in endosperm tissue from developing kernels, it remains specific to the origin of explant, since no transcripts are detected in leaf-tissue-derived suspension cultures. Transcript sizes are identical to those in developing seed endosperm tissue. Furthermore, accurate transcription initiation of the 10- and 27-kDa zein genes is observed by S1 nuclease mapping. Protoplasts isolated from endosperm cultures are capable of expressing foreign genes when transfected by electroporation. It is demonstrated here that the 5' flanking sequences of the 10- and 27-kDa zein genes are capable of promoting chloramphenicol acetyltransferase (CAT) gene expression in these transfected protoplasts. The observations described here show that these maize endosperm cultures can be used as an efficient homologous system to study transcriptional regulation of zein genes.

#### 2. Introduction

Zeins are the alcohol-soluble fraction of storage proteins in maize (Zea mays). They constitute more than 50% of the total endosperm proteins at seed maturity. Zeins consist of a group of heterologous hydrophobic proteins, which are classified according to their molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) into subclasses with Ms of

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27, 22, 19, 16, 15, and 10kDa (8, 9). Based on structural similarities, they are also classified into  $\alpha$ - (22 and 19 kDa),  $\beta$ - (15kDa),  $\gamma$ - (16 and 27kDa), and  $\delta$ - (10kDa) zeins (10). Zeins are encoded by a complex multigene family of over 100 gene members (11-13) and regulated in a tissue- and developmental stage-specific manner. Their expression is confined to triploid (3n) endosperm tissue and starts at a specific stage [around 12 days after pollination (DAP)] during endosperm development (14). Furthermore, the onset of elevated zein gene expression coincides with the genome amplification process starting at this particular stage in endosperm development (15).

The relatively long life cycle of maize plants and the lack of a readily attainable maize transformation method have rendered the study of zein gene regulation rather difficult. To circumvent these problems, zein gene regulation has been studied by stable or transient transformation experiments in heterologous systems including dicot plant species, mononuclear alga Acetabularia mediterranean, and yeast. Recently, protoplasts isolated from endosperm tissue at 10 DAP have been utilized as a homologous system in a transient expression study, and functional activity of a 19-kDa zein gene promoter was demonstrated (47). However, isolation of a large quantity of protoplasts was only feasible with the endosperm tissue at developmental stages prior to 11 DAP.

On the other hand, maize endosperm cells have been successfully cultured, and unlike many other cultured plant cells, they remain differentiated. They maintain the syntheses of starch (48) anthocyanins (49-51), and

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zeins (52, 53), which are characteristic of developing endosperm cells. As for the zein synthesis, accumulation in protein bodies has been observed in cultured maize endosperm cells as in endosperm cells of developing kernels, indicating the similarity in cellular processes between the two systems (52, 54). RNA slot-blot analysis confirmed transcription of the 27-kDa zein genes in short-term cultured endosperm cells (55).

Such maize endosperm cultures have been initially characterized here for the expression of zein genes. The regulation of  $\gamma$ - and  $\delta$ -class zein genes is of particular interest. Unlike  $\alpha$ -zeins, which are encoded by a large multigene family of 25-50 gene members (12, 13, 56),  $\gamma$ - and  $\delta$ -zeins, together with  $\beta$ -zeins, are encoded by genes present in few copies (20, 44, 57, 58), which simplifies molecular analysis of their gene regulation. The work presented here shows tissue-specific expression of genes encoding 10-, 15-, and 27-kDa zeins in cultured maize endosperm cells. Accurate transcription initiation of the 10- and 27-kDa zein genes was observed. Using a transient expression system with protoplasts isolated from these cultured endosperm cells, the promoter function of the 5' flanking sequences of the 10- and 27-kDa zein genes was demonstrated. The findings here indicate that these maize endosperm cells are an efficient homologous system for studying the regulation of zein genes.

### 3. Materials and methods

#### a. Maize cell cultures

Endosperm tissue cultures were established from developing endosperm tissue (13 DAP) from maize inbred

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line A636 (Maize Stock Center, University of Illinois, Urbana). Callus cultures were initiated from the excised endosperm tissues on a semisolid medium consisting of Murashige and Skoog (59) (MS) salts supplemented with 0.15 g/l L-asparagine, 0.5 mg/l thiamine HCl, 3% (w/v) sucrose, and 0.8% (w/v) Bacto agar, pH 5.8. One to two months after the culture initiation, calli proliferating on the surface of the explants were transferred into a liquid medium (the same medium as above, except that agar is omitted). Endosperm cell cultures were maintained thereafter in liquid suspension for more than 1 year, while being subcultured routinely every 7 days. Suspension cultures derived from immature leaf tissues of germinating seedlings of maize inbred line Black Mexican Sweet (BMS) were used as a control. They were maintained in a liquid medium consisting of MS salts kept in the dark at 26°C in a growth room. A636 endosperm and BMS suspension cultures were shaken on horizontal shakers at 160 and 230 rpm, respectively.

20 **b. RNA blot analysis**

Total RNA was isolated from maize plant tissues and cultured cells according to the procedure described by Das et al. (22). Northern and slot-blot analyses were performed according to the procedures described by Cruz-Alvarez et al. (28). The amounts of RNA loaded are described in the figure legends. The 10-kDa zein gene probe used was a 450-bp NcoI-XbaI fragment of cDNA clone p10kz-1 (constructed according to the method of Kirihara et al. (21)) from inbred line W22 (21). The 15-kDa zein gene probe used was a 932-bp EcoRI-BamHI fragment of genomic subclone pGEMZ14 (constructed according to the method of Pederson et al. (60)) from inbred line W64A (60). The 27-kDa zein gene probe used was a 1.2-kb SphI-

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Sall fragment (obtained according to the method of Das and Messing (44)) of a genomic subclone from inbred line W22 (61). The 17S rDNA probe used was a 1.5-kb SstI fragment of the M13 clone 6L-1 (constructed according to the method of Messing et al. (62)) (62). All probes were labeled with  $^{32}\text{P}$ -dCTP by nick-translation (63). Hybridization intensities on the autoradiograms for the slot-blots were quantitated densitometrically with a Joyce-Loebl Chromoscan-3 densitometer at 530 nm.

c. Determination of the 5' ends of the 10- and 27-kDa zein mRNA by S1 nuclease mapping

Transcription initiation sites were determined by S1 nuclease mapping (64) for the 10- and 27-kDa zein genes in both developing (16 DAP) endosperm cells. Probes for the two zein genes were prepared from genomic subclones (constructed according to the method of Kirihara et al. (20)) by isolating DNA fragments which extend from the 5' flanking sequences preceding them (Fig. 3A). The probe for the 10-kDa zein mRNA was prepared from a genomic subclone derived from inbred line BSSS53. A 1,238-bp HindIII-BanI fragment was initially isolated, and the terminal phosphates were removed with calf intestine alkaline phosphatase (CIP, Boehringer Mannheim). It was 5' end-labeled with  $\gamma$ - $^{32}\text{P}$ -ATP by T4 polynucleotide kinase. Subsequently, the radiolabeled fragment was digested with AvaII and a 188-bp 5' end-labeled AvaII\*-BanI fragment was purified by electrophoresis in an 8% polyacrylamide gel. The probe for the 27-kDa zein mRNA was prepared from a genomic subclone derived from inbred line W22. A 474-bp HpaII fragment was isolated and 5' end-labeled with  $\gamma$ - $^{32}\text{P}$ -ATP by T4 polynucleotide kinase. Subsequently, it was digested with RsaI and a 343-bp fragment purified by electrophoresis in an 8% polyacrylamide gel.



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Strand-specific probes (20,000 cpm) were precipitated with ethanol together with total RNA isolated from developing (16 DAP) endosperm tissue (25  $\mu$ g) or from cultured endosperm cells (50-75  $\mu$ g). They were resuspended in 30  $\mu$ l of hybridization buffer consisting of 0.4 M NaCl, 40 mM PIPES (pH 6.4), 1 mM EDTA, and 80% (v/v) formamide. They were then heat-denatured at 70°C for 10 minutes and allowed to anneal for 12 hours at 46-50°C. After hybridization, the reaction mixtures were incubated with 300  $\mu$ l of S1 nuclease (500 units/ml) in 0.25 M NaCl, 50 mM sodium acetate (pH 4.6), 4.5 mM ZnSO<sub>4</sub>, and 20  $\mu$ g/ml salmon sperm DNA as a carrier, at 37°C for 30 minutes. The S1 nuclease reaction was terminated and the nucleic acids were precipitated by the addition of 10  $\mu$ l of 500 mM EDTA, 50  $\mu$ l of 4 M ammonium acetate, 1  $\mu$ l of tRNA (10 mg/ml), and 1 ml of ethanol. The products of the S1 nuclease reactions were analyzed together with Maxam and Gilbert (16) sequencing reactions of the probes on 6% polyacrylamide gels containing 8 M urea.

#### d. Construction of chimeric genes

Plasmid pFFCAT was used for construction of chimeric genes containing 5' flanking sequences of the 10- and 27-kDa zein genes. pFFCAT contains a 777-bp TaqI fragment of the CAT coding sequence (from -30 to +747 with respect to the ATG initiation codon) cloned into the SalI site of pFF19 (17) (Fig. 4A). The 5' flanking sequences of the 10-kDa zein gene were isolated from genomic subclone pG10BH7 (constructed according to the method of Kirihara et al. (20)) as a 1,118-bp HindIII-BamHI fragment spanning from -1,076 to +42 with respect to the cap site, ATC, as described in the Results section of Part III. The BamHI site has been created by converting the nucleotides AG to TC (located at +43 and +44 with respect

to the cap site) by site-directed mutagenesis. Subsequently, it was cloned into the HindIII/BamHI sites of pFFCAT, thus replacing the CaMV35S promoter. It was designated as pZ10(-1076/+42) CAT (Fig. 4A). For the 27-kDa zein gene, a 1,103-bp PvuI fragment spanning from -1042 to +61 (with respect to the cap site, ATC, as described in the Results section, Part III) was isolated from a genomic subclone. The fragment was blunt-ended with T4 DNA polymerase and subcloned into the HindIII and XbaI, and cloned into the HindIII/XbaI sites of pFFCAT, replacing the CaMV35S promoter. It was designated as pZ27(-1042/+61) CAT (Fig. 4A). As a negative control in the transient expression experiments, a promoter-less CAT construction ( $\phi$ -CAT, Fig. 4A) was prepared by digesting pFFCAT with HindIII and SmaI. The protruding HindIII end was made blunt by Klenow reaction and the plasmid was religated.

#### e. Protoplast isolation

Protoplasts were isolated enzymatically from endosperm suspension cells. Approximately 20-30 g (fresh weight) of suspension cells was collected in sterile, 50-ml disposable tubes and washed once with CPW solution (65) containing 0.65 M D-mannitol. They were incubated in a total volume of 100 ml of enzyme mixture consisting of 3% (w/v) cellulysin (Calbiochem), 1% (w/v) macerase (Calbiochem), and 0.25% (w/v) cellulase (Worthington) in CPW solution with 0.65 M D-mannitol (pH 5.4), with gentle shaking (50 rpm) on a horizontal shaker for 10-12h at room temperature in the dark. Isolated protoplasts were purified by passing the digestion mixture successively through 140 $\mu$ m and 74 $\mu$ m stainless steel sieves. When required, protoplasts were further purified by the sucrose [21-23% (w/v)] flotation method (65). Purified

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protoplasts were washed twice with CPW solution with 0.65 M D-mannitol, and the protoplast density was determined with a hemacytometer.

f. Electroporation of endosperm protoplasts

The isolated endosperm protoplasts were washed once with  
5 phosphate buffer saline (PBS) containing 0.65 M D-mannitol and resuspended in the same buffer at a final density of  $2-3 \times 10^6$  protoplasts/ml. The protoplasts were kept on ice until electroporation. The covalently closed circular plasmid DNA containing the chimeric gene  
10 construct was suspended in 500  $\mu$ l of PBS containing 0.65 M D-mannitol in a 2.9-ml disposable spectrophotometer cuvette (Ultra-VU cuvettes-micro, Fisher). Five hundred microliters of endosperm protoplast suspension was added to the cuvette and mixed thoroughly with the plasmid DNA  
15 suspension. While keeping the cuvette on ice, electroporation was carried out at 250 V, 600  $\mu$ F using a stainless steel electrode with a gap width of 4.5 mm (PDS, Inc.). The electro-suspension culture medium was supplemented with 0.65 M D-mannitol in a plastic petri  
20 dish. The dish was sealed with parafilm and incubated at 25°C in the dark. To standardize the electroporation efficiency for different constructs, 10  $\mu$ g of pFFGUS plasmid DNA (17), containing the  $\beta$ -glucuronidase reporter gene fused to the CaMV35S gene promoter (with a  
25 duplicated enhancer) and terminator, was cotransfected in each electroporation experiment.

g. Chloramphenicol acetyltransferase (CAT) and  $\beta$ -glucuronidase (GUS) enzyme assays

30 At the end of a 44- to 48-h culture period, transfected protoplasts were collected in a 15-ml conical disposable tube. Subsequently, they were resuspended in 400  $\mu$ l of

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250 mM TRIS (pH 7.0), 10 mM EDTA in a 1.5-ml microfuge tube and homogenized with a disposable pellet pestle (Kontes Scientific Glassware/Instruments). The homogenate was spun down in a micro-centrifuge for 10 minutes at 4°C, and the protein concentration in the supernatant was determined using a BioRad Protein Assay Kit. CAT and GUS assays were carried out according to the procedures described by Malmberg et al. (66) and Jefferson et al. (67), respectively. The CAT activity was quantitated by measuring in a scintillation counter the radioactivity of the silica gel spots containing the <sup>14</sup>C-labeled chloramphenicol and acetylated forms. GUS activity was determined fluorimetrically, using 4-methylumbelliferyl glucuronide (MUG) as a substrate. Fluorescence was measured with a Perkin-Elmer Fluorescence Spectrometer (model LSD-3B), with excitation at 365 nm and emission at 455 nm.

#### 4. Results

##### a. Tissue-specific expression of the 10-, 15-, and 27-kDa zein genes in cultured endosperm cells

RNA blot analysis of total RNA isolated from different tissues of maize plants showed endosperm-specific expression of the genes encoding the 10-, 15-, and 27-kDa zeins (Fig. 1). Transcripts of these three zein genes were also detected in total RNA isolated from suspension culture cells derived from developing endosperm tissue. To rule out the possibility that the observed zein gene expression in cultured cells had arisen from aberrant gene regulation during tissue culture manipulation, the expression of these zein genes in BMS suspension culture cells derived from leaf tissue was examined. Transcripts of the three zein genes could not be detected in the BMS

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cells (Fig. 1). No gross rearrangement of these gene loci was detected in the genomic DNA isolated from cultured endosperm cells by Southern blot analysis (data not shown). These observations indicate that the expression of the 10-, 15-, and 27-kDa zein genes in these maize endosperm cultures is regulated in a tissue-specific manner.

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However, in cultured endosperm cells the expression levels were reduced for all three zein genes as compared with those in the developing endosperm tissue (16 DAP). For better quantitation of the reduction in zein gene expression levels, RNA was further analyzed by slot blots (Fig. 2). When hybridization to the three zein gene probes was standardized by hybridization to the 17S rDNA probe, it was found that the reductions of RNA levels for the 10-, 15-, and 27-kDa zein genes were about 199-, 22-, and 46-fold, respectively. Nevertheless, expression of these zein genes in the cultured cells is significant, since no transcripts were detected in the embryos (16 DAP) or roots or leaves of young plants when the same amounts of total RNA from these tissues were used for the analysis. (Fig. 1).

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b. Accurate transcription initiation of the 10- and 27-kDa zein genes in cultured endosperm cells

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In the RNA blot analysis described previously (Fig. 1), a discrete major transcript was identified for the 10-, 15-, and 27-kDa zein genes in both cultured endosperm cells and developing endosperm tissues. The sizes of these transcripts appeared to be identical in both systems, suggesting accuracy in transcription of zein genes in the cultured endosperm cells. To further analyze the accuracy of transcription, transcription

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initiation sites were determined for the 10- and 27-kDa zein genes by S1 nuclease mapping. Since genomic subclones and DNA sequence data are not available for the 10- and 27-kDa zein genes in inbred line A636, the probes for S1 nuclease mapping experiments were prepared by utilizing the genomic subclones derived from inbred lines BSSS53 and W22 for the 10- and 27-kDa zein genes, respectively (Fig. 3A). Thus, total RNA isolated from developing endosperm tissue (16-18 DAP) of inbred lines BSSS53 and W22 were also used in the experiments as controls for the 10- and 27-kDa zein gene probes, respectively.

As shown in Fig. 3B, 2-4 major bands were detected in the S1 protection assays for both 10- and 27-kDa zein transcripts with total RNA derived from developing endosperm tissue as well as from cultured endosperm cells of inbred line A636. The sizes of these bands are identical for the two systems, demonstrating accurate transcription initiation for the two zein genes in cultured endosperm cells. Their sizes are also identical to those for the control RNAs, indicating that the DNA sequences around the 5' coding and flanking regions of the 10- and 27-kDa zein genes are conserved between inbred lines BSSS53 and A636, and between W22 and A636, respectively. Comparison of the protected bands with the Maxam-Gilbert (16) sequencing reactions of the corresponding probes mapped the transcription initiation sites to positions -61/-60 and -71/-70/-69/-68 with respect to the ATG initiation codons on the genomic clones, for the 10- and 27-kDa zein genes, respectively. These transcription initiation sites are marked by the consensus trinucleotide ATC, where a majority of transcripts initiate at nucleotide A. Transcription

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initiation at or around this consensus trinucleotide has also been observed for the  $\alpha$ -zein (68) and  $\beta$ -zein genes (69, 70), indicating the conservation of transcription initiation sites for many zein genes.

c. 5' Flanking sequences of the 10- and 27-kDa zein genes promote chimeric gene expression in cultured endosperm cells

The observations of tissue-specific expression and accurate transcription initiation of the 10- and 27-kDa zein genes in cultured endosperm cells have led to testing whether or not these cultured cells could be used to study transcriptional regulation of zein genes. An efficient method is established here for isolating a large quantity ( $0.5-2 \times 10^6$ ) of viable protoplasts from endosperm suspension cultures by enzymatic digestion of cell walls. Although isolated protoplasts seldom undergo active cell division, they remain viable for more than 2 weeks in culture. Thus, a transient gene expression system was developed by introducing chimeric gene constructions into these protoplasts by electroporation.

For the construction of chimeric genes, 5' flanking DNA sequences of approximately 1.1 kb in size were isolated from genomic clones of the 10- and 27-kDa zein genes derived from inbred lines BSSS53 and W22, respectively. They were fused to the coding sequence of the E. coli CAT gene fused to the 3' terminator sequences derived from the CaMV35S gene (Fig. 4A). Promoterless CAT gene construct  $\phi$ -CAT, and pFFCAT containing the CaMV35S promoter with a duplicated enhancer element (17), were used as negative and positive controls, respectively, for the transient gene expression experiments (Fig. 4A).

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As shown in Fig. 4B, the CaMV35S gene promoter yielded a high level of CAT gene expression in transfected endosperm protoplasts at an input plasmid amount of 25  $\mu$ g when assayed 48 hours after transfection. On the other hand, levels, of CAT gene expression promoted by the 5' flanking sequences of the 10- and 27-kDa zein genes at this input plasmid level were much lower (data not shown). To enhance the detection of CAT gene expression produced by the 5' flanking sequences of zein genes, a higher amount of plasmid DNA was required per electroporation.

Electroporation with 150-200  $\mu$ g of plasmid DNA resulted in easily detectable levels of CAT gene expression in transfected protoplasts (Fig. 4B). CAT gene expression driven by the 5' flanking sequences of these zein genes was significant when compared with the negative controls, where neither plasmid DNA nor the same amount of promoter-less  $\phi$ -CAT construction was electroporated (Fig. 4B). The promoter activity of the 5' flanking sequences was higher, by six- to seven-fold, for the 10-kDa zein gene than for the 27-kDa zein gene, as determined by standardizing the CAT activity yielded by these 5' flanking sequences to the GUS activity derived from the CaMV35S promoter in cotransfected pPFGUS plasmid. These observations have demonstrated that protoplasts isolated from cultured endosperm cells are capable of transiently expressing chimeric genes driven by the 5' flanking sequences of the 10- and 27-kDa zein genes.

## 5. Discussion

Cultured maize endosperm cells are unique in that they remain differentiated rather than becoming "dedifferentiated" as do most cultured plant cells. An



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endosperm tissue-specific characteristic maintained in these cultured endosperm cells is the synthesis of zein proteins (52, 53). Biochemical and cellular processes involved in zein synthesis and accumulation in protein bodies in cultured endosperm cells follow, to some extent, those taking place in developing endosperm tissue (52, 54). The RNA analysis presented here reveals that genes encoding the 10-, 15-, and 27-kDa zeins are expressed in endosperm cultures of maize inbred line A636. Expression of these zein genes represents the maintenance of the differentiated state of explants rather than their reactivation during tissue culture manipulation, since these genes are not expressed in leaf tissue-derived BMS cultures. Furthermore, maintenance of accurate transcription of zein genes in endosperm cultures can be inferred from the following findings: 1) synthesis of a discrete major transcript for each zein gene; 2) sizes of the zein transcripts, which are identical to those in developing endosperm tissue; and 3) accurate transcription initiation sites for the 10- and 27-kDa zein genes.

Despite tissue-specific expression of the zein genes, their mRNA levels are drastically reduced in the maize endosperm culture. Reduction in the content of zein polypeptides has also been shown in maize endosperm cultures, where the alcohol-soluble protein fraction is less than 20% of the total proteins (53). Furthermore, the relative proportion of zeins in endosperm culture differs from that in developing endosperm tissue (53). While  $\alpha$ -zeins (19- and 22-kDa zeins) are the major (80-90%) constituent among zeins in developing endosperm tissue, they comprise only 10-15% in endosperm culture. In addition, accumulation of a large amount of the 27-kDa

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zein was also observed in endosperm culture, which is in agreement with our finding of a comparatively higher level of the 27-kDa zein mRNA in these cultures.

5 The reduced levels of zein polypeptides and transcripts in cultured endosperm cells raises a question concerning their developmental stage. Endosperm tissue in developing kernels consists of a population of heterogeneous cells with regard to their developmental stages (71). It is known that active zein synthesis starts around 10-12 DAP, when cell division ceases for most endosperm cells (72). Cessation of cell division occurs first in the cells present in the central part of the endosperm, while cells present at the region immediately beneath the aleurone (outer cell layer or subaleurone layer) remain meristematic (73, 74). The fact that the cultured endosperm cells, derived from endosperm tissue harvested at 13 DAP, remain active in cell division suggest that they are enriched in meristematic cells, typically present at the outer cell layer of the endosperm. There is predominant synthesis of  $\beta$ - and  $\gamma$ -zeins in these cells, while  $\alpha$ -zeins are synthesized at very low levels in the outer cell layer (75). Previous observation that the 27-kDa zein polypeptides are relatively abundant in cultured endosperm cells (53) supports the notion that these cultured cells represent the meristematic cells of the outer cell layer. We have observed that the 22-kDa zein gene expression in the endosperm culture was much more severely reduced than the 15- and 27-kDa zein gene expression (data not shown), which is also in agreement with the above notion.

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Based on the observations of tissue-specific expression and accurate transcription of zein genes in endosperm cultures, an efficient transient expression system has been established by utilizing these cultures. A large quantity of protoplasts can be isolated from cultured endosperm cells and transfected with foreign genes by electroporation. It was shown that the 1.1-kb 5' flanking sequences of the 10- and 27-kDa zein genes can promote expression of the CAT reporter gene in transfected endosperm culture protoplasts. The levels of CAT gene expression promoted by the 5' flanking sequences of these two zein genes are much lower than that driven by the CaMV35S promoter with a duplicated enhancer element. A large amount of plasmid DNA harboring these chimeric gene constructs is required in electroporation to obtain detectable levels of CAT activity, suggesting weak promoter activities of the 5' flanking sequences of these zein genes. Weak promoter activity of the 5' flanking sequences of the Z4 zein ( $\alpha$ -zein) gene has also been reported previously in transgenic tobacco plants (76). It is known that the endosperm cells that have ceased cell divisions continue an active DNA replication, leading to the increase in their C value up to 90°C by 16 DAP (15). Observations of weak promoter activity of the zein gene 5' flanking sequences, therefore, are not surprising if we consider that overexpression of zein genes in developing endosperm tissue may rely on the genome amplification process taking place during endosperm development. It is also interesting to note that the 5' flanking sequences of the 10-kDa zein gene have a higher promoter activity than those of the 27-kDa zein gene, which is converse to the levels of these two zein transcripts in cultured endosperm cells.

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In summary, maize endosperm cultures offer at least the following two advantages as a homologous model system to study endosperm-specific gene regulation. The first is that these cultures can be maintained in a laboratory throughout the year under a defined environmental condition, which circumvents a long waiting period and a large field or greenhouse space required for obtaining endosperm tissues from maize plants. The second is that a large quantity of protoplasts can be easily isolated from these cultures for transfection with chimeric gene constructions. In the absence of a readily attainable transformation method for maize plants at present, although a recent breakthrough (77) promises the use of such an approach in the future, these maize endosperm cultures provide a valuable system for studying endosperm-specific gene regulation.

#### IV. Genetic Transformation of Maize (Zea mays L.) Plants

Although genetic transformation, mainly mediated by Agrobacterium tumefaciens, has become a routinely used practice for many dicotyledonous plant species, it is still a difficult and laborious task for graminaceous crop species including maize. This is mainly due the inability of their somatic cells and protoplasts to undergo active cell divisions in culture and subsequent regeneration into whole plants. To overcome this problem, some improvements have been made in the selection of regenerable cell cultures (such as an embryogenic cell culture) as well as in transformation techniques. To date, a few gene transfer methods have been successfully used to generate transgenic maize plants. These methods are summarized below.

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1. Direct gene transfer method.

This method relies on the uptake of DNA into embryonic protoplasts isolated from embryogenic maize cell culture and the subsequent regeneration of whole plants. DNA uptake is induced by polyethylene glycol (PEG) or electroporation. A critical requirement for this method is the establishment of a highly regenerable maize cell culture. This method has been successfully used in the genetic transformation of maize plants. See, for example, Gordon-Kamm et al. (77).

2. Particle discharge (acceleration) method.

This method utilizes the bombardment of intact cells and tissues with DNA-coated microprojectiles (78). An advantage of this method is its application for intact cells, especially for meristematic cells, thus, eliminating a laborious step for plant regeneration from single protoplasts. This method has been successfully used to generate transgenic maize plants. See, for example, Fromm et al. (79).

3. Agroinfection method.

The Agrobacterium-mediated gene transfer method, originally believed to be applicable only to dicotyledonous plant species, has been proven to be capable of transforming monocotyledonous plant species including maize. This method relies on the ability of Agrobacterium tumefaciens, a soil bacterium, to transfer and integrate a part of DNA sequence (T-DNA) on its plasmid (Ti plasmid) into the plant genome. Successful genetic transformation of maize plants can be achieved by the infection of meristematic cells at the shoot apex with Agrobacterium and subsequent development of whole plants. See, for example, Gould et al. (80).

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### Materials and Methods

RNA isolated from maize plant tissue and cultured cells was analyzed by Northern blot analysis. Total RNA was isolated from endosperm (16 DAP), root and leaf tissues of an A636 maize plant, as well as from A636 endosperm and leaf tissue-derived BMS cultures. Five micrograms of RNA sample from each tissue were fractionated in a formaldehyde-agarose gel, transferred onto a filter, and hybridized to a <sup>32</sup>P-labelled O2 cDNA probe. The results from this experiment, shown in Figure 5, demonstrate that the endosperm-specific expression of the O2 gene is maintained in the A636 endosperm culture.

The effect of the O2 overexpression on two zein promoters was tested using chimeric constructs, a diagrammatic representation of which is provided in Figure 6, panel A. A diagrammatic representation of the O2 overexpression constructs which are cotransfected with the zein promoter-GUS constructs shown in Figure 6, panel A, is provided in Figure 6, panel B. Relative GUS activities derived from the transiently transformed maize endosperm protoplasts which have been cotransfected with the zein promoter-GUS and the O2 overexpression constructs are shown in Figure 6, panel C.

Chimeric constructs, diagrammatically represented in Figure 7, panel A, were used to test the effect of O2 overexpression on the O2-binding cis sequence from the Z-4 promoter. These constructs were cotransfected into maize endosperm protoplasts together with the O2 overexpression cassettes shown in Figure 6, panel B. The relative CAT activities derived from the transiently transformed maize endosperm protoplasts are shown in Figure 7, panel B.

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The plasmid pUM5010 (ATCC Accession No. 68644) contains a zein protein-encoding sequence, a promoter region, and an exogenous O<sub>2</sub>-binding region. The cloning steps used to construct the pUM5010 plasmid are illustrated in Figure 8.

5 The response of zein promoters to an exogenously added phytohormone, abscisic acid (ABA) is shown in Figure 9. Other than the trans-regulation by Opaque-2 proteins, it is also found that ABA can differentially regulate promoter function of zein genes. As seen in Figure 9,  
10 exogenously added ABA (50 $\mu$ M) enhances the promoter activity of the 27-kDa zein gene while it suppresses that of the 10-kDa zein gene. In addition, the promoter activity of the CaMV35S promoter is not affected by ABA. It is speculated here that different classes of  
15 endogenous zein genes may respond differentially to the endogenous ABA level in the kernel which is known to increase during seed maturation. Endosperm protoplasts transfected with each construct (described in the Brief Description of Figure 9) were cultured for two days in  
20 the absence (-) or presence (+) of 50  $\mu$ M ABA before enzymatic assays were performed.

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What is claimed is:

1. A method of obtaining corn seeds or kernels having a methionine content of greater than 1.39 percent by weight of the total amino acid composition of the corn seeds or kernels, which comprises crossing a paternal inbred corn line containing the Zpr10/(22) locus with a maternal inbred corn line lacking the Zpr10(22) locus and selecting for F1 hybrid seeds containing methionine at greater than 1.39 percent by weight of the total amino acid composition of the F1 hybrid seeds or kernels.
2. A method of claim 1, wherein the paternal inbred corn line is BSSS-53.
3. A method of claim 1, wherein the maternal inbred corn line is M017, W23 or W22.
4. A method for providing greater than 36 percent of the methionine nutritional requirements of poultry which comprises feeding the poultry corn having a methionine content of at least 1.39 percent by weight of the total amino acid composition of the corn.
5. A method of claim 4, wherein the corn has a methionine content of at least 3.8 percent.
6. A method of claim 4, wherein the poultry are chickens.

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7. A method of claim 4, wherein the corn is in the form of seeds or kernels.
8. A method of claim 4, wherein the corn is in the form of cornmeal.
- 5 9. A method of improving the growth performance of poultry which comprises feeding the poultry corn having a methionine content of at least 3.8 percent by weight of the total amino acid composition of the corn.
- 10 10. A corn plant resulting from a genetic cross comprising high zein protein-containing seeds having a methionine content of at least 1.39 percent by weight of the total amino acid composition of the seeds.
- 15 11. A corn plant of claim 10, wherein the seeds have a methionine content of at least 3.8 percent.
- 20 12. A corn plant of claim 10, wherein the genetic cross is a reciprocal cross between a Zpr10/(22)-containing maize inbred and a normal female maize inbred lacking Zpr10/(22).
- 25 13. A corn plant of claim 12, wherein the Zpr10/(22)-containing inbred is BSSS-53.
- 30 14. A corn plant of claim 12, wherein the normal female maize inbred is M017, W23 or W22.
- 35 15. A recombinant nucleic acid molecule consisting essentially of (1) a sequence encoding a zein

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protein, (2) a sequence which when present in the molecule is capable of functioning as a promoter of transcription of the sequence encoding the zein protein, and (3) an exogenous sequence capable of (a) enhancing the functioning of the promoter sequence, (b) stabilizing the transcription product of the zein protein-encoding sequence, or (c) enhancing the translation of the transcription product of the zein protein-encoding sequence, the promoter sequence and the exogenous sequence being so positioned with respect to the sequence encoding the zein protein that the zein protein is expressed when the recombinant nucleic acid molecule is introduced into a suitable host cell.

16. A recombinant nucleic acid molecule of claim 15, wherein the sequence encoding the zein protein is a naturally occurring zein gene.

17. A recombinant nucleic acid molecule of claim 15, wherein the naturally occurring zein gene is a maize zein gene.

18. A recombinant nucleic acid molecule of claim 17, wherein the maize zein gene is a high-methionine maize zein gene.

19. A recombinant nucleic acid molecule of claim 18, wherein the high-methionine maize zein gene is a 10kDa high-methionine maize zein gene.

20. A recombinant nucleic acid molecule of claim 15, wherein the exogenous sequence comprises an O<sub>2</sub>-binding region of a maize 22kDa promoter.

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21. A recombinant nucleic acid molecule of claim 20,  
wherein the exogenous sequence is a multimer of O2-  
binding regions of maize 22kDa promoters.
22. A recombinant nucleic acid molecule of claim 21,  
wherein the multimer of O2-binding regions of maize  
22kDa promoters comprises 5 copies of the O2-binding  
region.
23. A recombinant nucleic acid molecule of claim 22,  
wherein the molecule is designated pUM5010 (ATCC  
Accession No. 68644).
24. A recombinant nucleic acid molecule of claim 15,  
wherein the exogenous sequence comprises an ABA  
regulatory element.
25. A recombinant nucleic acid molecule of claim 24,  
wherein the exogenous sequence is a multimer of ABA  
regulatory elements.
26. A recombinant nucleic acid molecule of claim 25,  
wherein the multimer of ABA regulatory elements  
comprises of 5 copies of the ABA regulatory element.
27. A recombinant nucleic acid molecule of claim 15,  
wherein the exogenous sequence comprises the 3'  
region of the B gene of the 27kDa maize gene.
28. A recombinant nucleic acid molecule of claim 27,  
wherein the 3' region of the B gene of the 27kDa  
maize gene is fused to the 3' end of the sequence  
encoding the zein protein.

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29. A method of increasing the concentration of zein protein in a plant, which comprises treating the plant so as to incorporate the nucleic acid molecule of claim 15 into the genome of the plant.
30. A method of claim 29, wherein the plant is maize.
- 5 31. A method of claim 29, wherein the plant is rice.
32. A method of claim 29, wherein the plant is soybean.
- 10 33. A method of claim 29, wherein the plant is alfalfa.
34. A method of claim 29, wherein the plant is barley.
35. A method of claim 29, wherein the plant is wheat.
- 15 36. A method of claim 29, wherein the incorporation of the nucleic acid molecule comprises microinjecting the molecule into cells of the plant.
- 20 37. A method of claim 29, wherein the incorporation of the nucleic acid molecule comprises firing the molecule with a particle gun into cells of the plant.
- 25 38. A method of claim 29, wherein the incorporation of the nucleic acid molecule comprises contacting embryonic culture cells of the plant with the molecule.
- 30 39. A genetically engineered corn plant having high zein protein-containing seeds and a methionine content of at least 1.39 percent by weight of the total amino
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acids present in the seeds comprising the recombinant nucleic acid of claim 15.

- 5 40. A genetically engineered corn plant of claim 39, wherein the methionine content of the high zein protein-containing seeds is at least 3.8 percent by weight of the total amino acids present in the seeds.
- 10 41. A method of determining whether an exogenous nucleic acid molecule will be expressed in maize endosperm tissue which comprises introducing the exogenous nucleic acid molecule into cultured maize endosperm protoplasts suspended in a suitable buffer; culturing the resulting maize endosperm protoplasts containing the exogenous nucleic acid molecule; and  
15 detecting expression of the nucleic acid molecule by the maize endosperm protoplasts so as to thereby determine whether the nucleic acid molecule will be expressed in maize endosperm tissue.
- 20 42. A method of claim 41, wherein the exogenous nucleic acid molecule is the recombinant nucleic acid molecule of claim 15.
- 25 43. A method of claim 41, wherein the introduction of the exogenous nucleic acid molecule comprises performing electroporation on the protoplasts in the presence of the exogenous nucleic acid molecule.
- 30 44. A method of claim 41, wherein the detection of expression comprises isolating RNA from the cultured protoplasts containing the nucleic acid molecule;



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and determining the presence of RNA transcribed from the nucleic acid molecule in the RNA so isolated.

- 5 45. A method of claim 41, wherein the detection of expression comprises performing an enzyme assay on cultured protoplasts containing the nucleic acid molecule, wherein the nucleic acid molecule encodes an enzyme having detectable activity, and detecting this activity of the enzyme.
- 10 46. A method of claim 45, wherein the enzyme is chloramphenicol acetyltransferase (CAT).
47. A method of claim 45, wherein the enzyme is  $\beta$ -glucuronidase (GUS).
- 15 48. A purified antibody specific for a maize high-methionine protein.
- 20 49. An antibody of claim 48, wherein the maize high-methionine protein is the maize 10kDa high-methionine protein.
- 25 50. A method of determining the level of high-methionine protein produced by a maize strain which comprises preparing a protein-containing sample from the maize strain; contacting the sample with the antibody of claim 48 under conditions such that the antibody complexes with any high-methionine protein present in the sample for which the antibody is specific; and determining the amount of high-methionine protein present in any resulting complex.
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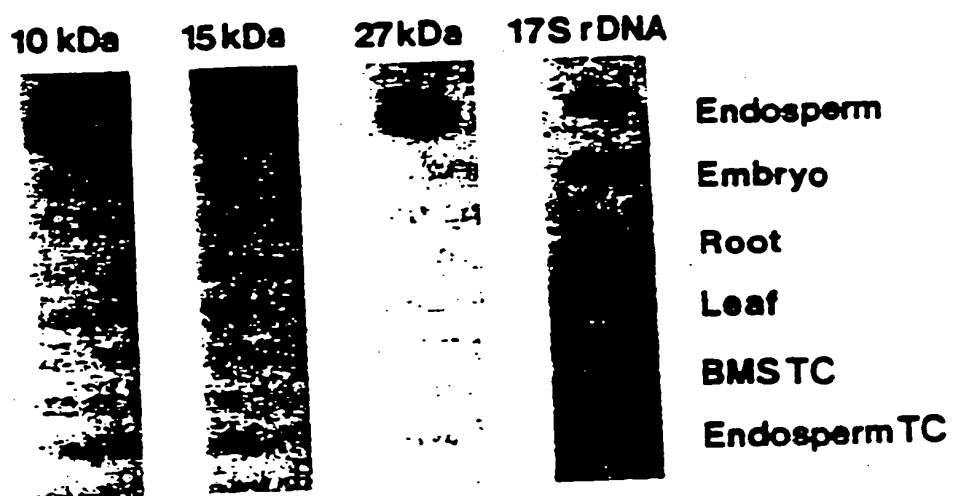
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FIGURE 1



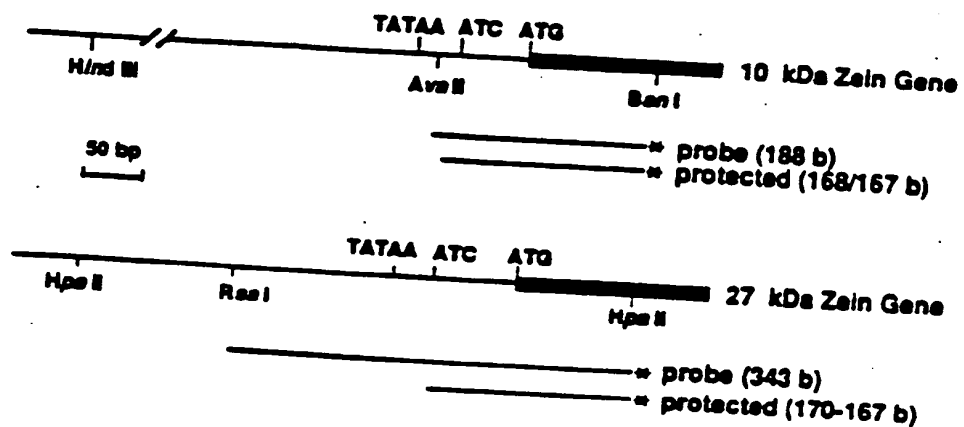
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FIGURE 2



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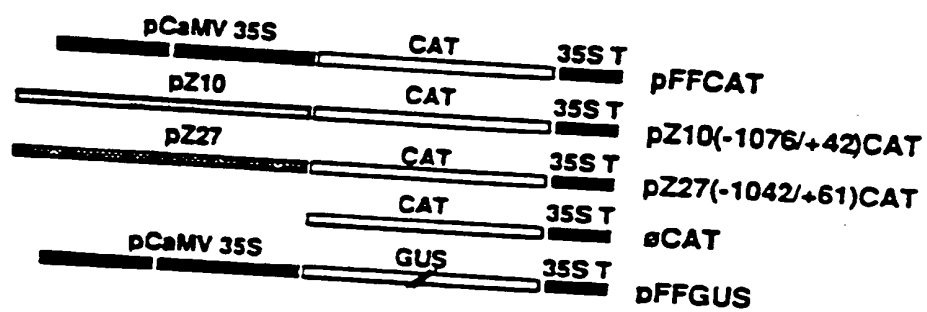
FIGURE 3A





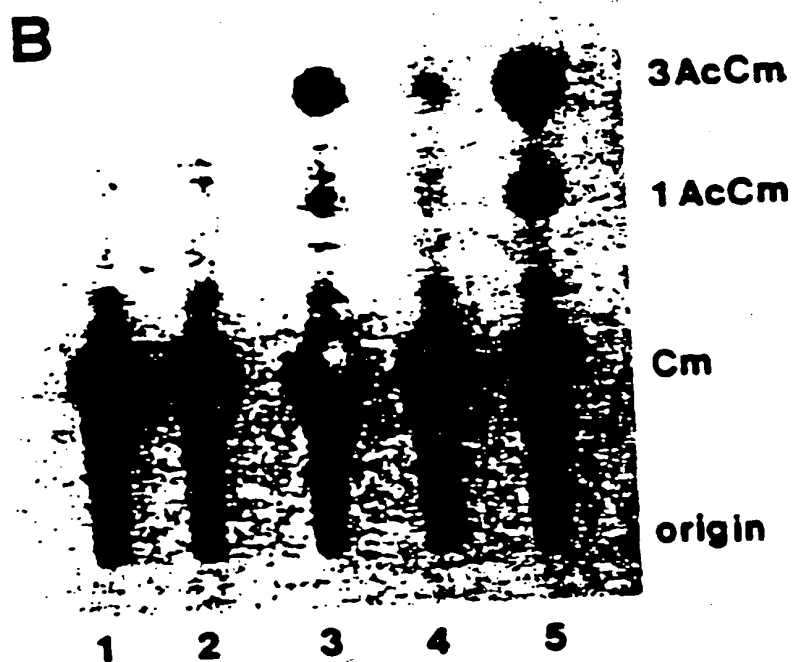
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FIGURE 4A



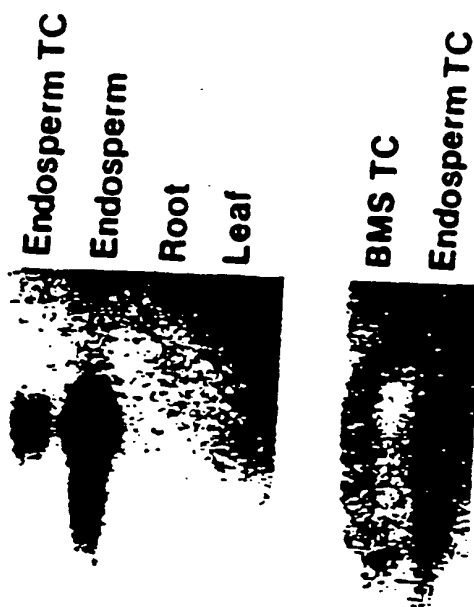
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FIGURE 4B



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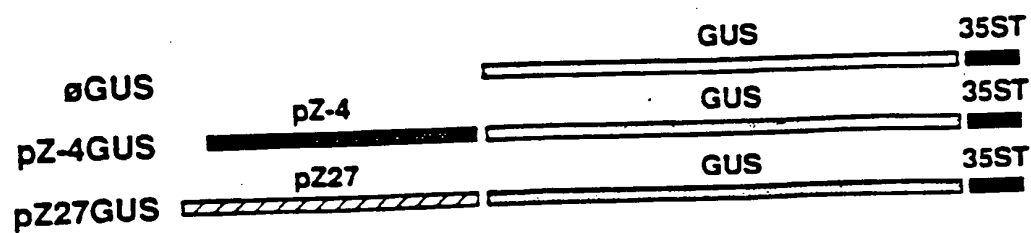
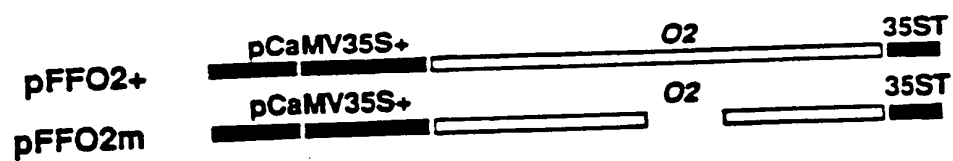
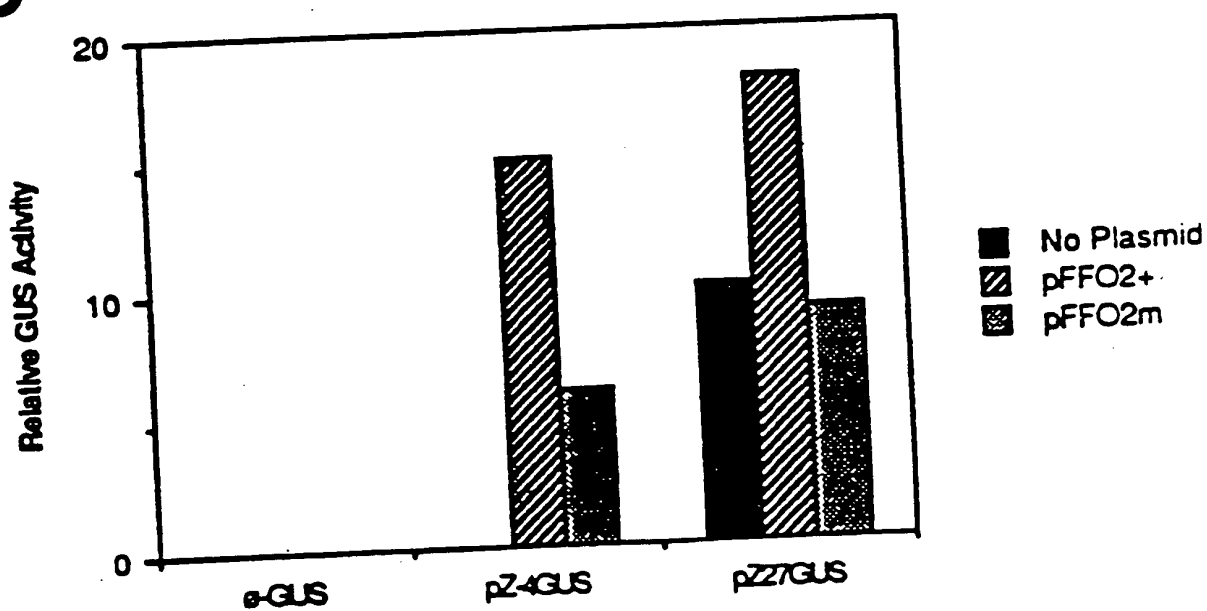
FIGURE 5





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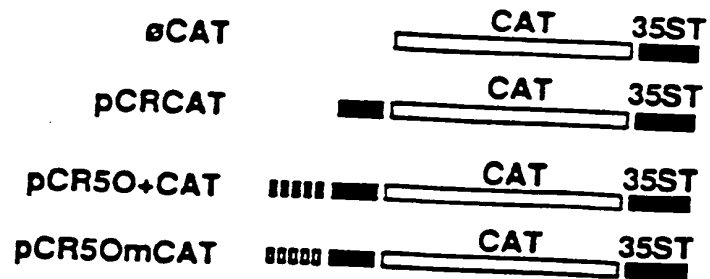
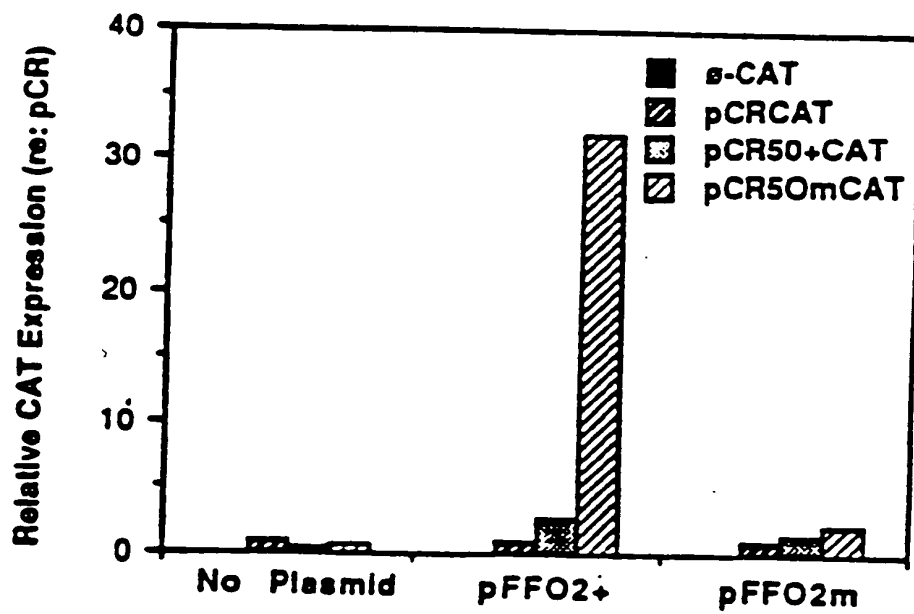
FIGURE 6

**A****B****C**

**A**

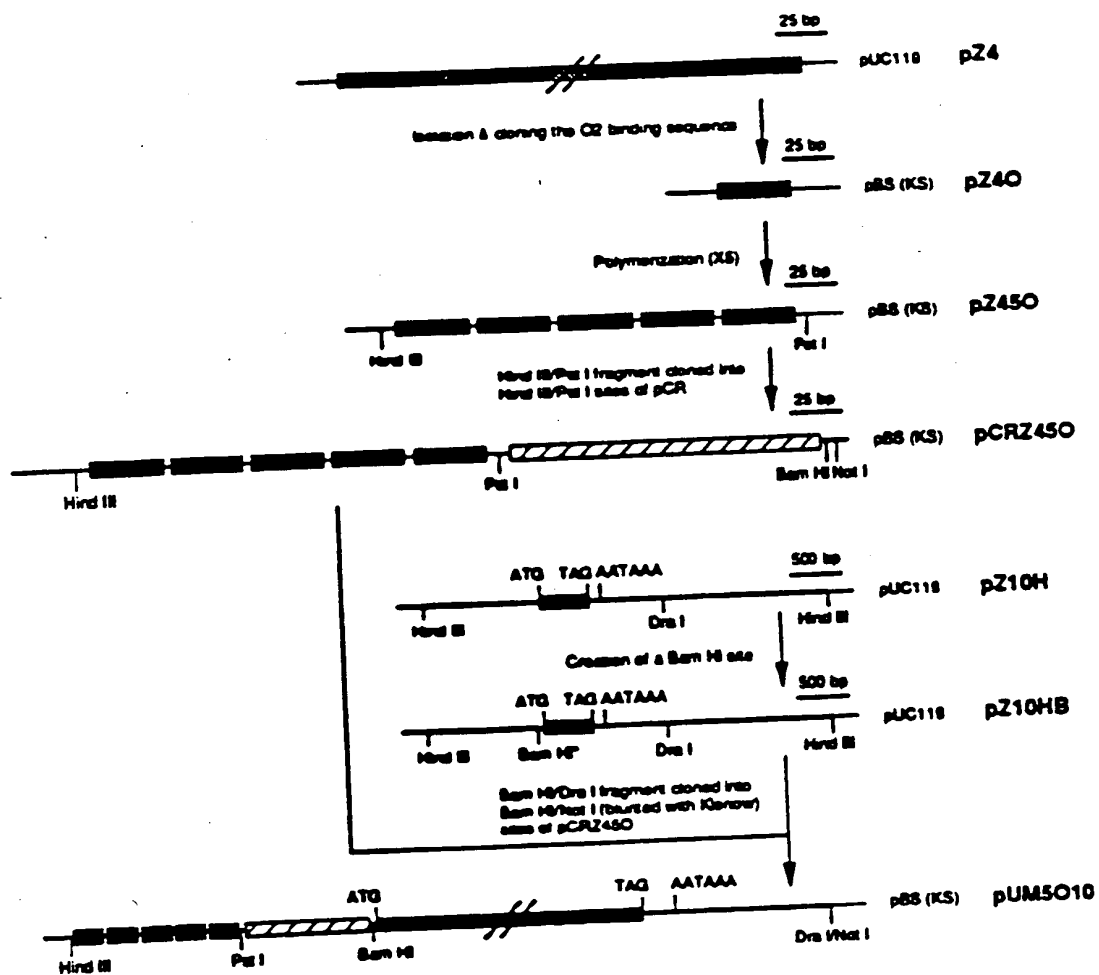
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FIGURE 7

**B**

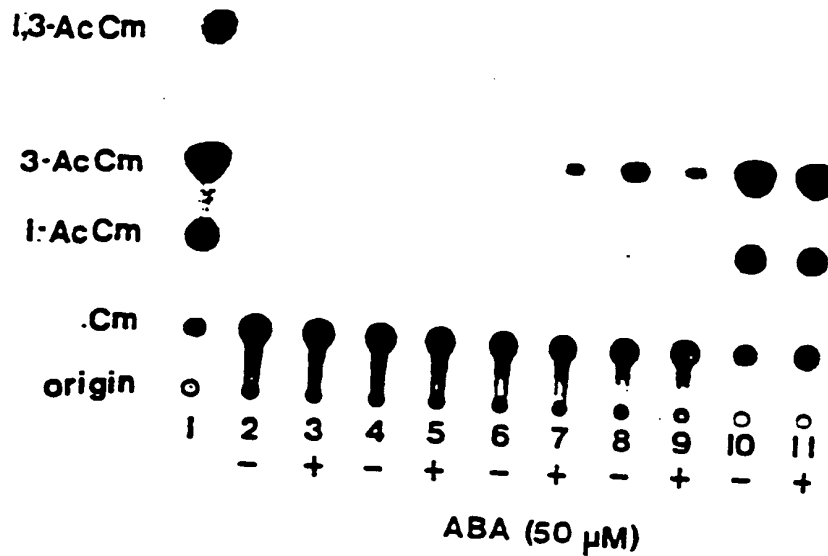
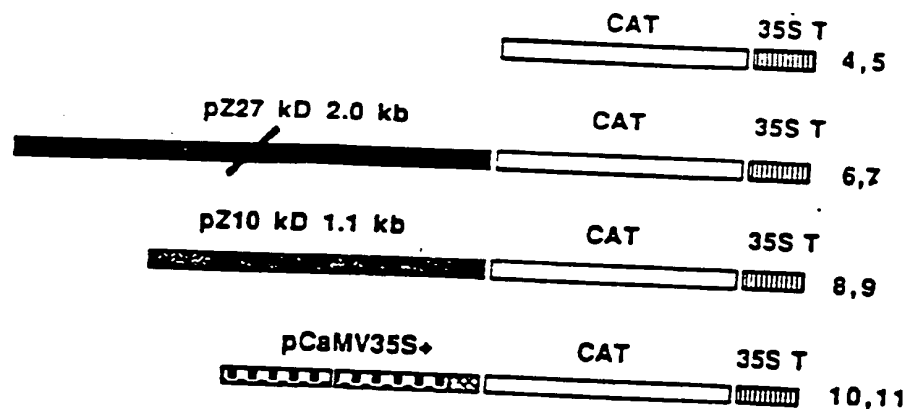
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FIGURE 8



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FIGURE 9



# INTERNATIONAL SEARCH REPORT

In...national application No.  
PCT/US92/09433

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : Please See Extra Sheet.

US CL : 47/58; 426/630; 800/205, 250; 435/172.3, 240.47; 530/387; 436/547

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 47/58; 426/630; 800/205, 250; 435/172.3, 240.47; 530/387; 436/547; 935/6, 9, 41, 43, 53, 56, 57

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog/BIOSIS

search terms: opaque 2, o2, abscissic acid, ABA, gene, transcript, induc?, element, wheat, transform?

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Trends in Biotechnology, Volume 8, issued June 1990, Susan B. Altenbach et al., "Manipulation of Methionine-Rich Protein Genes in Plant Seeds", pages 156-160, especially page 156.	4-9
Y	Crop Science, Volume 21, issued 1981, R. L. Phillips et al., "Seedling Screening for Lysine-Plus-Threonine Resistant Maize", pages 601-607, especially pages 605-606.	1-3, 10-14, 39, 40
Y	Gene, Volume 71, issued 1988, J. A. Kiriara et al., "Isolation and Sequence of a Gene Encoding a Methionine-Rich 10-kDa Zein Protein from Maize", pages 359-370, especially pages 360 and 362.	1-3, 10-14, 18, 19, 23, 39, 40
Y	Plant Physiology, Volume 88, issued 1988, J. D. Williamson et al., "The Synthesis of a 19 Kilodalton Zein Protein in Transgenic <u>Petunia</u> Plants", pages 1002-1007, especially page 1002.	15-40
Y	Science, Volume 236, issued 5 June 1987, R. Kay et al., "Duplication of CaMV 35S Promoter Sequences Creates a Strong Enhancer for Plant Genes", pages 1299-1302, especially page 1301.	15-40



Further documents are listed in the continuation of Box C.



See patent family annex.

- \* Special categories of cited documents:
- \*A\* document defining the general state of the art which is not considered to be part of particular relevance
- \*E\* earlier document published on or after the international filing date
- \*L\* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

T

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

&

document member of the same patent family

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01 February 1993

Date of mailing of the international search report

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Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/09433

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, Volume 87, issued January 1990, R. J. Schmidt et al., "Maize Regulatory Gene Opaque-2 Encodes a Protein with a "Leucine-Zipper" Motif That Binds to Zein DNA", pages 46-50, especially page 50.	20-23
Y	U.S. A, 4,983,518 (Schaffner et al.) 08 January 1991, col. 2, lines 34-49.	21-23, 26
Y	Science, Volume 250, issued 12 October 1990, M. J. Gultinan et al., "A Plant Leucine Zipper Protein That Recognizes an Abscissic Acid Response Element", pages 267-271, especially page 269.	24-26
Y	Molecular and Cellular Biology, Volume 7, No. 12, issued December 1987, O. P. Das, "Allelic Variation and Differential Expression at the 27-Kilodalton Zein Locus in Maize", pages 4490-4497, especially page 4491.	27, 28
Y	The Plant Cell, Volume 2, issued July 1990, W. J. Gordon-Kamm et al., "Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants", pages 603-618, see entire article.	30, 37-40
Y	Bio/Technology, Volume 6, issued September 1988, K. Toriyama et al., "Transgenic Rice Plants After Direct Gene Transfer Into Protoplasts", pages 1072-1074, see entire article.	31
Y	Bio/Technology, Volume 6, issued August 1988, D. E. McCabe et al., "Stable Transformation of Soybean (Glycine Max) By Particle Acceleration", pages 923-926, see entire article.	32, 37
Y	Plant Cell Reports, Volume 5, issued 1986, M. Deak et al., "Transformation of <u>Medicago</u> by <u>Agrobacterium</u> Mediated Gene Transfer", pages 97-100, see entire article.	33
Y	G. Lycett and D. Grierson (editors), "Genetic Engineering of Crop Plants", published 1990 by Butterworths (London), pages 230-238, especially page 237.	34
Y	Plant Science, Volume 72, issued 1990, D. Hess et al., "Transformation Experiments by Pipetting <u>Agrobacterium</u> into the Spikelets of Wheat ( <u>Triticum aestivum</u> L.), pages 233-244, especially page 234.	35
Y	Theoretical and Applied Genetics, Volume 75, issued 1987, G. Neuhaus et al., "Transgenic Rapeseed Plants Obtained by the Microinjection of DNA into Microspore-Derived Embryoids", pages 30-36, see entire article.	36
Y	Nature, Volume 319, issued 27 February 1986, M. E. Fromm et al., "Stable Transformation of Maize After Gene Transfer By Electroporation", pages 791-793, especially page 791.	41-47
Y	Journal of Biotechnology, Volume 2, issued 1985, J. M. Norrander et al., "Manipulation and Expression of the Maize Zein Storage Proteins in <u>Escherichia Coli</u> ", pages 157-175, especially page 161.	48-50

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/09433

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

A01H 1/00, 1/02, 5/00; A23K 1/00; A61K 35/14; C07K 3/00, 15/00; C12N 15/00; G01N 33/53

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

I. Claims 1-3 and 10-14, drawn to a method of crossing inbred corn lines to obtain high-methionine (HM) seeds, classified in Class 47, subclass 58, for example.

II. Claims 4-9, drawn to a method for feeding HM feed to poultry, classified in Class 426, subclass 630, for example.

III. Claims 15-40, drawn to a recombinant maize gene encoding HM zein, and to transformation of plants with said gene, classified in Class 800, subclass 205, for example.

IV. Claims 41-47, drawn to a method to determine whether a cloned gene is expressible in maize endosperm protoplasts, classified in Class 435, subclass 240.47, for example.

V. Claims 48-49, drawn to a purified antibody specific for a HM zein protein, classified in Class 530, subclass 387, for example.

VI. Claim 50, drawn to a method for using zein-specific antibody to quantitate the amount of HM protein produced by a maize strain, classified in Class 436, subclass 547, for example.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/09433

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☒  
☐

The additional search fees were accompanied by the applicant's protest.  
No protest accompanied the payment of additional search fees.